UNIVERSIDAD COMPLUTENSE DE MADRID

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Departamento de Sanidad Animal

TESIS DOCTORAL

Identificación y caracterización de un mecanismo emergente de resistencia a aminoglucósidos las metiltransferasas del ARNr 16S

Identification and characterization of an emergent aminoglycoside resistance mechanism the 16S rRNA methyltransferases

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List of Abbreviations

AGs. Aminoglycosides

AMEs. Aminoglycoside modifying enzymes

AN. Genbank accession number

BLEE. Beta-lactamasa de espectro extendido

CFU. Colony forming units

CPB. Carbapenemase-producing bacteria

DOS. Deoxystreptamine

ESBL. Extended-spectrum beta-lactamase

EUCAST. European Committee on Antimicrobial Susceptibility Testing

MDR. Multi-drug-resistant

MIC. Minimum inhibitory concentration

MLST. Multi-Locus Sequence Typing

MRSA. Meticillin-resistant-staphylococcus aureus

MT. methyltransferase

PFGE. Pulsed-field get electrophoresis

UFC. Unidades formadoras de colonias

WHO. World Health Organization

RESUMEN

El descubrimiento de los antibióticos a comienzos del siglo XX supuso una de las mayores revoluciones para la medicina moderna. Sin embargo, la euforia de haber superado el reto de las enfermedades infecciosas producidas por bacterias no duró mucho. Poco tiempo después de empezar a utilizarse los antibióticos, las bacterias comenzaron a desarrollar distintas formas de resistencia, hecho que se fue incrementando con el aumento del uso de los antibióticos. En la actualidad, la resistencia a antibióticos es una carrera de fondo en la que el descubrimiento de nuevas moléculas antibacterianas se ve superado por la velocidad de desarrollo de nuevos mecanismos de resistencia por parte de las bacterias. Por ello, la resistencia a antibióticos constituye un grave problema sanitario, tanto de Salud Pública como de Sanidad Animal, a escala mundial.

Los aminoglucósidos son una familia de antibióticos de gran relevancia clínica para el tratamiento de infecciones producidas por bacterias gram-positivas y gram-negativas, tanto en medicina humana como en medicina veterinaria. Su acción se basa en su unión a la subunidad ribosómica bacteriana 30S, en concreto al ARNr 16S en la región decodificante del sitio A, donde se comprueba la veracidad de la traducción. Como consecuencia la bacteria acumula proteínas erróneas lo que conlleva la muerte celular. La resistencia bacteriana a los aminoglucósidos se debe casi siempre a la producción de enzimas capaces de acetilar (acetiltransferasas), fosforilar (fosfotransferasas), o adenilar (nucleotidiltransferasas) el antibiótico inactivándolo. Cada enzima modificadora tiene especificidad por determinados aminoglucósidos, y aunque pueden suponer un problema en la práctica clínica, el nivel de resistencia que confieren no suele ser muy elevado. Adicionalmente se han descrito otros mecanismos de resistencia a aminoglucósidos, como la reducción de la concentración intracelular del antibiótico, o la modificación de la diana por mutaciones. No obstante, estos mecanismos no son muy significativos desde el punto de vista clínico.

En 2003, sin embargo, se descubrió un nuevo mecanismo de resistencia a aminoglucósidos en bacterias gram-negativas. Este mecanismo consiste en la adición de un grupo metilo por parte de una enzima metiltransferasa en el ARNr 16S, lo cual bloquea la unión del aminoglucósido al ribosoma. Esta metiltransferasa, denominada ArmA (*Aminoglycoside resistance methyltransferase A*), guarda homología con las metiltransferasas codificadas en el cromosoma de las bacterias ambientales productoras de aminoglucósidos. Estas bacterias, del orden Actinomycetales, metilan el ARNr 16S para evitar su propio suicidio por el antibiótico que producen. Además, las metiltransferasas del ARNr 16S adquiridas por bacterias gram-negativas se caracterizan por conferir un elevado nivel de resistencia a prácticamente todos los aminoglucósidos disponibles en la práctica clínica. Es por ello que su descubrimiento supuso una alarma para la Salud Pública, ante la posible pérdida de eficacia de una de las familias de antibióticos más relevantes tanto en medicina humana como medicina veterinaria.

Por este motivo, los objetivos de esta Tesis Doctoral son la identificación de metiltransferasas de resistencia a aminoglucósidos en bacterias gram-negativas de distinto origen, y la caracterización molecular de dichas cepas así como de las plataformas genéticas implicadas en su transmisión.

Este trabajo consta de cuatro publicaciones con las cuales hemos contribuido a un mayor conocimiento sobre la presencia y la diseminación de las metiltransferasas del ARNr 16S en bacterias gram-negativas de origen humano, animal y alimentario.

Por un lado, hemos detectado por primera vez la presencia de ArmA, la metiltransferasa más prevalente hasta ahora, en una cepa aislada de alimento. Esta cepa se aisló en la Isla de Reunión y se identificó como *Salmonella enterica* 1.4,12:i:−, una variante monofásica de *S. enterica* Typhimurium. Se observó que portaba la isla genómica 1 de *Salmonella* (SGI1), y tres plásmidos que denominamos pB1008, pB1009, y pB1010. Los plásmidos pB1008 y pB1010 se transfirieron por conjugación a una cepa receptora de laboratorio, *E. coli* K802N. Las frecuencias de conjugación de pB1008 y pB1010 fueron 4×10^{-4} y 9×10^{-4} UFC por donadora, respectivamente, aunque ambos plásmidos se transfieren

conjuntamente con una frecuencia de 10^{-5}. El análisis de distintos determinantes de resistencia reveló la presencia de los genes bla_{CMY-2} en pB1008, y *armA* y $bla_{CTX-M-3}$ en pB1010. La técnica de digestión con nucleasa S1 y PFGE permitió comprobar que el tamaño de los plásmidos pB1008 y pB1010 era 50 kb y 110 kb, respectivamente. Además, la clasificación de los plásmidos siguiendo un protocolo de tipado por PCR reveló que pB1010 pertenecía al grupo de incompatibilidad IncP, mientras que pB1008 y pB1009 no eran tipables siguiendo esta metodología. Por otro lado, se desarrolló un sistema de 9 PCRs para amplificar el Tn*1548*, la plataforma genética en la cual *armA* se había visto ubicado previamente, y se comprobó la presencia de *armA* en un derivado de Tn*1548*.

En otra de las publicaciones hemos identificado ArmA en cepas de *S. enterica* serovars Thompson y Worthington de origen clínico aisladas en Reino Unido. En este caso observamos que *armA* está localizado en dos plásmidos conjugativos, pB1015 en *S.* Thompson, y pB1016 en *S.* Worthington. pB1015 es un plásmido IncHI2 de 245 kb, mientras que pB1016 es un plásmido IncA/C de 170 kb, que, además de *armA*, codificaba el gen de la beta-lactamasa CMY-2, y el gen de una nueva BLEE de tipo VEB que denominamos bla_{VEB-5}. Este último confiere resistencia a cefalosporinas de 3ª generación y a aztreonam. El mapeo por PCR de Tn*1548* nos permitió comprobar cómo distintas variantes genéticas de este transposón parecen estar implicadas en la diseminación de *armA*.

La tercera publicación comprende un estudio de la presencia de estas metiltransferasas en aislados clínicos de India. Se analizaron 1000 aislados consecutivos de enterobacterias, observándose altos niveles de resistencia a aminoglucósidos en el 14% (140 aislados). Identificamos ArmA, RmtB, y RmtC por PCR en el 46%, 20%, y 27% de estos 140 aislados. Mediante ensayos de clonaje y posterior secuenciación, descubrimos un nuevo miembro de la familia de las metiltransferasas del ARNr 16S que confieren resistencia a aminoglucósidos, RmtF, en 34 de estas cepas. Esta nueva metiltransferasa comparte la mayor identidad en aminoácidos con RmtD (46%), y confirmamos mediante espectrometría de masas MALDI que confiere alto nivel de resistencia a aminoglucósidos mediante la adición de un grupo metilo en posición G1405 del

ARNr 16S. *rmtF* se encontró localizado en distintos plásmidos, la mayoría de ellos transferibles, y el 59% de las cepas productoras de *rmtF* portaban la carbapenemasa NDM. Además, *rmtF* se detectó en seis cepas clínicas productoras de NDM-1 aisladas en Reino Unido.

Por último, hemos detectado ArmA por primera vez en cepas procedentes de animales de compañía. Se trata de siete *Klebsiella pneumoniae* aisladas de perros y gatos en España. Tras su caracterización mediante MLST comprobamos que todas ellas son *K. pneumoniae* ST11, un clon epidémico adaptado a humanos y previamente descrito en España asociado a determinantes de resistencia emergentes, lo cual muestra el alarmante papel que pueden representar los animales de compañía como reservorios de estos mecanismos de resistencia. En estos aislados, *armA* está en pB1025, un plásmido IncR de 50 kb. Esta es la primera descripción de un plásmido IncR en bacterias aisladas de animales de compañía. Dichos aislados también co-producen las β-lactamasas DHA-1 y SHV-11, y el determinante de resistencia a fluoroquinolonas QnrB4.

En conclusión, esta Tesis Doctoral contribuye a un mayor conocimiento sobre uno de los mecanismos de resistencia a antibióticos más novedosos, las metiltransferasas del ARNr 16S, así como analiza las interrelaciones humano-animal-alimento en su dinámica propagación.

SUMMARY

The discovery of antibiotics at the beginning of the 20th century constituted one of the biggest revolutions in modern medicine. However, the euphoric feeling of overcoming the challenge of bacterial-caused diseases did not last. Shortly after the initiation of antibiotics use, bacteria started to develop diverse ways of resistance, so the higher usage of antibiotics, the higher incidence of resistant bacteria. Nowadays, antibiotic resistance is a race where the development of bacterial resistance mechanisms is faster than the discovery of new antibacterial molecules. Not for nothing, antibiotic resistance poses a serious global problem, for both Public and Animal Health.

Aminoglycosides are a family of antibiotics very important in human and veterinary medicine for the treatment of infections caused by gram-positive and gram-negative bacteria. They bind to the bacterial 16S rRNA in the 30S ribosomal subunit, specifically at the point where the fidelity of translation is verified. This binding causes the accumulation of erroneous proteins, leading to the cell death. Bacterial resistance to these antibiotics is generally due to the production of enzymes that are able to acetylate (acetyltransferases), phosphorylate (phosphotransferases), or adenylate (nucleotidyltransferases) the antibiotic molecule, thus making it inactive. Every modifying enzyme shows specificity for certain aminoglycosides, and even though they can represent a problem in clinical settings, they do not usually confer high levels of resistance. Additionally, other aminoglycoside resistance mechanisms have been described, such as the reduction of the intracellular concentration of antibiotic, or the target modification by mutation. Nevertheless, these mechanisms are not highly significant from a clinical point of view.

In 2003, however, a new aminoglycoside resistance mechanism was discovered in gram-negative bacteria. It consists on the addition of a methyl group to the 16S rRNA by a methyltransferase enzyme, which blocks the aminoglycoside

binding to the ribosome. This protein, named ArmA (*Aminoglycoside resistance methyltrasnferase A*), shares homology with those methyltransferases encoded in the chromosome of aminoglycoside-producing environmental bacteria. These bacteria, belonging to the Actinomycetales, methylate the 16S rRNA in order to avoid their suicide by the antibiotic that they generate. Furthermore, acquired 16S rRNA methyltrasferases in gram-negative bacteria confer high-level resistance to almost all aminoglycosides available in the clinical practice. Therefore, their discovery posed a threat for the Public Health, facing the likely loss of efficacy of one of the most relevant antibiotic families for human and veterinary medicine.

For this reason, the objectives of this Doctoral Thesis are the identification and molecular characterization of aminoglycoside resistance methyltransferases in gram-negative bacteria from diverse origins, as well as the analysis of the genetic platforms involved in their transmission.

This work is constituted by four publications that have contributed to a deeper knowledge about the presence and spread of the 16S rRNA methyltransferases in gram-negative bacteria from humans, animals, and food.

On one side, we have detected for the first time ArmA, the most prevalent methyltransferase thus far, in a strain isolated from food. This strain, isolated in La Reunion Island, was identified as *Salmonella enterica* 1.4,12:i:−, a monophasic variant of *S. enterica* Typhimurium. It was observed the presence of *Salmonella* genomic island 1 (SGI1), as well as three plasmids named pB1008, pB1009, and pB1010. Plasmids pB1008 and pB1010 were transferred by conjugation to a laboratory recipient strain, *E. coli* K802N. The conjugation frequencies for pB1008 and pB1010 were 4×10^{-4} and 9×10^{-4} per donor CFU, respectively, and both plasmids were cotransferred at a frequency of 10^{-5} per donor CFU. The analysis of several resistance determinants revealed the presence of $bla_{\text{CMY-2}}$ in pB1008, and *armA* and $bla_{\text{CTX-M-3}}$ in pB1010. S1-nuclease digestion and PFGE allowed to determine that plasmids pB1008 and pB1010 were 50 kb and 110 kb in size, respectively. Moreover, plasmid PCR-based replicon typing showed that pB1010 belongs to the IncP incompatibility group, whereas pB1008 and pB1009 are non-typable following this methodology. Furthermore, we developed a system based on

9 overlapping PCRs to amplify the Tn*1548*, the genetic platform where *armA* had been previously identified, and it was observed to be embedded in a Tn*1548* derivative.

In another publication we have identified ArmA in clinical *S. enterica* serovars Thompson and Worthington isolated in the United Kingdom. In this case, *armA* is located on two conjugative plasmids, pB1015 in *S.* Thompson, and pB1016 in *S.* Worthington. pB1015 is an IncHI2 plasmid of 245 kb, whereas pB1016, sized 170 kb, belongs to the IncA/C group, and, besides *armA*, it bears $bla_{\text{CMY-2}}$, and a novel VEB ESBL gene, called $bla_{\text{VEB-5}}$. The latter confers resistance to 3rd generation cephalosporins and aztreonam. Tn*1548* mapping showed how different variants of this transposon are involved in the dissemination of *armA*.

The third publication comprises a prevalence study of this methyltransferases in clinical isolates from India. We analyzed 1000 consecutive *Enterobacteriaceae* isolates, and we observed high-level aminoglycoside resistance in the 14% (140 isolates). Out of these, we identified by PCR ArmA, RmtB, and RmtC in the 46%, 20%, and 27%, respectively. After cloning experiments and subsequent sequencing, we discovered a new member of the aminoglycoside resistance 16S rRNA methyltransferases family, RmtF, in 34 strains. This novel methyltransferase shares the highest amino acid identity with RmtD (46%), and we confirmed by MALDI mass spectrometry that it confers high-level aminoglycoside resistance by specifically adding a methyl group at position G1405 in the 16S rRNA. *rmtF* was found located on diverse plasmids, most of them transferable by electroporation, and 59% of *rmtF*-producing strains also carried NDM carbapenemase. In addition, *rmtF* was detected in six clinical NDM-producing strains isolated from the UK.

Finally, we have described ArmA for the first time in bacteria from companion animals. It was identified in seven *K. pneumoniae* isolates from dogs and cats in Spain. MLST characterization showed that all of them are *K. pneumoniae* ST11, a human epidemic clone previously reported in Spain in association with emerging resistance determinants. This is worrisome as it implies

companion animals may be a reservoir for these resistance mechanisms. In these isolates, armA is borne by pB1025, and IncR plasmid of 50 kb. To the best of our knowledge, this is the first report of an IncR plasmid in bacteria from pets. The isolates also co-produce β-lactamases DHA-1 and SHV-11, and the fluoroquinolone resistance determinant QnrB4.

Overall, this Doctoral Thesis has contributed to a better understanding of an emerging antibiotic resistance mechanism, the 16S rRNA methyltransferases, as well as it has analyzed the interrelations human-animal-food in their rapid spread.

INTRODUCTION

CHAPTER 1. Aminoglycoside antibiotics

1.1- Historical aspects

After the discovery of penicillin as an antibacterial substance produced by *Penicillium rubens*, and attributed to Alexander Fleming in 1928, the aminoglycosides were the first important therapeutic agents produced by bacterial fermentation. In 1944, Albert Schatz and Selman Waksman discovered streptomycin by screening different cultures of soil bacteria for the presence of inhibitory activity against *M. tuberculosis* (Schatz *et al*., 1944). This molecule was produced by the actinobacterium *Streptomyces griseus,* and it became the first antibiotic effective against tuberculosis. For this discovery S. Waksman was awarded the Nobel Prize in Medicine in 1952. The second aminoglycoside, neomycin, was reported in 1949, but its toxicity issues coupled with the emerging resistance to streptomycin gave rise to additional aminoglycosides discoveries in the 1950s and early 1960s (Jana and Deb, 2006), including kanamycin (produced by *Streptomyces kanamyceticus*) and gentamicin (isolated from *Micromonospora purpurea*). But the increasing appearance of resistant strains producing enzymes capable of modifying the antibiotic molecule led to the search for novel synthesized aminoglycoside derivatives (Kondo, 1999). Thus, in 1975, dibekacin was approved for therapeutic use as the first designed semisynthetic aminoglycoside. Arbekacin, a kanamycin B semisynthetic derivative, is the most recent aminoglycoside introduced for antibacterial therapy in the 1990s in Japan (Jana and Deb, 2006).

Most aminoglycosides discovered to date are produced by Actinobacteria of either the genus *Streptomyces* or *Micromonospora*. Aminoglycosides that are derived from *Streptomyces* are named with the suffix –mycin (e.g. streptomycin,

neomycin or kanamycin) whereas those derived from *Micromonospora* are named with the suffix –micin (e.g. gentamicin or sisomicin).

1.2- Mechanism of action

The major target of aminoglycosides is the bacterial 30S subunit (Figure 1). They bind to the A-site (Aminoacyl site) tRNA acceptor which is part of the 16S rRNA, leading to a loss of translational accuracy and subsequently, to the cell death (Jana and Deb, 2006). Biochemical and molecular biological approaches, as well as studies based on nuclear magnetic resonance (NMR) and X-ray crystal structures, have identified the basis for aminoglycoside binding to bacterial ribosomes (Magnet and Blanchard, 2005).

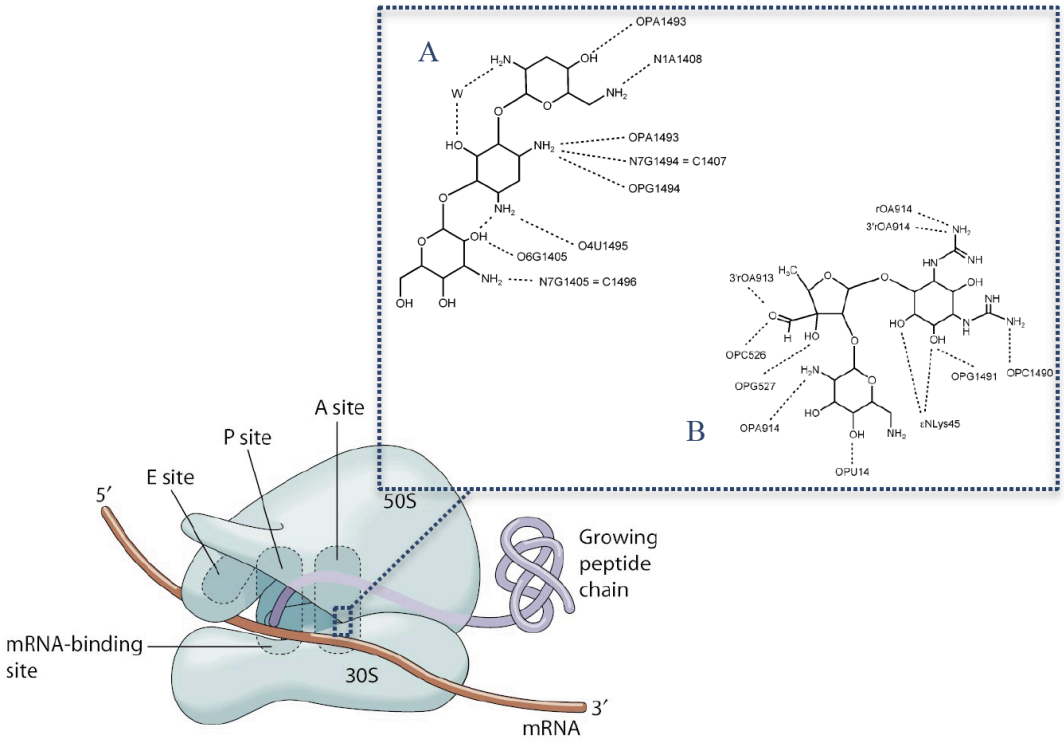

Figure 1. Schematic representation of the bacterial ribosome and an enlarged view of the aminoglycosides binding to the A site, showing the interactions between the antibiotic molecule and the respective residues. A: tobramycin (2-DOS), B: streptomycin (no 2-DOS). Adapted from Magnet and Blanchard, 2005.

Although aminoglycosides bind electrostatically to the outer membrane of gram-negative bacteria or to the exterior of the cell wall of gram-positive bacteria, in a non-energy dependent process, their uptake and diffusion across the cytoplasmic membrane requires energy generated from an oxygen-dependent process, called energy dependent phase I (EDP-I) (Taber *et al*., 1987). As a consequence, these antibiotics are less active in anaerobic conditions. Aminoglycoside uptake is a self-promoted process that requires a proton-motive force and involves a drug-induced disruption of Mg^{2+} bridges between adjacent lipopolysaccharide molecules (Mingeot-Leclerq *et al*., 1999). Once in the cytosol, aminoglycosides bind to the A-site in the small subunit of the ribosomes through an energy-dependent process called energy dependent phase II (EDP-II) (Magnet and Blanchard, 2005). The A site is comprised of, amongst others, a portion of helix 44. As it has been extensively described, the fidelity of translation depends mainly on the correct formation of the initiation complex, which involves the mRNA codon and the appropriate tRNA anticodon, and the subsequent proofreading during the elongation step. When a cognate tRNA-mRNA complex is formed, residues A1492 and A1493 are flipped out from helix 44, and this configuration allows the discrimination between correct or erroneous complexes (Carter *et al.*, 2000). Aminoglycoside binding takes place in a pocket formed by various residues of helix 44 which are universally conserved nucleotides A1492 and A1493, and the prokaryotic-specific nucleotide A1408. This aids in their selective activity against the bacterial as opposed to the eukaryotic ribosome (Jana and Deb, 2006).

It has been biochemically elucidated that when a 2-deoxystreptamine (2-DOS) aminoglycoside binds to this pocket, it causes a flip out of residues A1492 and A1493 similar to that which occurs when there is a cognate mRNA-tRNA complex. Therefore, there is no discrimination between cognate and non-cognate complexes, which disrupts the translation at the elongation level leading to the accumulation of aberrant proteins. These may be inserted into the cell membrane, altering the permeability and even increasing the aminoglycoside transport (Carter *et al*., 2000).

Regarding the specific mechanism of atypical aminoglycosides (non 2-DOS), the interaction of three of them (streptomycin, spectinomycin, and hygromycin B) with the 30S subunit has been studied at a biochemical level. Streptomycin binds to the A site, however, in contrast to the 2-DOS-aminoglycosides, it does not interact only with residues from helix 44 but also with residues from adjacent domains. It had been previously proposed that an interaction between helix 27 and helix 44 leads to two different ribosomal conformations and the equilibrium of which could be involved in the translational proofreading. Therefore, the binding of streptomycin appears to interfere with that balance. The binding of both spectinomycin and hygromycin B to the 30S subunit inhibits the translocation of the peptidyl-tRNA from the A site to the P site (Peptidyl site), although hygromycin B also causes miscoding at a lower level than DOS-aminoglycosides (Carter *et al*., 2000).

Besides the 30S ribosomal subunit as the major target of aminoglycosides, it has been recently shown that some aminoglycosides, such as gentamicin and neomycin, bind to helix 69 of the 23S rRNA in the large subunit. Binding at this site inhibits the ribosomal recycling process, a necessary step in which the two subunits separate from each other after the termination phase of protein synthesis (Wang *et al.*, 2012).

1.3- Properties and nomenclature

The broad-spectrum of aminoglycosides as well as their pharmacokinetics and potency is tightly related to their chemical properties. They are metabolically stable so they are excreted unmodified by the organism. Aminoglycosides are hydrophilic molecules, relatively insoluble in lipids, and they show an increased activity under alkaline conditions. These characteristics explain their poor absorption from the gut and the minimal penetration of the blood brain barrier. Aminoglycosides are positively charged (cationic), thus, they are able to bind to different cell membranes and intracellular anionic molecules (phospholipids, DNA, RNA). Nevertheless, this cationic nature contributes to their toxicity at physiological pH (Vincens and Westhof, 2002).

1.3.1- Chemical structure

The term "aminoglycoside" is given due to the general chemical structure of this group of antimicrobial agents. The backbone consists of an aminocyclitol ring that can be saturated with amine and hydroxyl substitutions, and it is linked to one or more amino sugars through glycosidic linkages (Benvetiste and Davies, 1973). In the case of spectinomycin, there is no amino sugar linked to the ring, therefore, various scientists have suggested that this group of agents should be described by the term "aminocyclitols" instead of "aminoglycosides" (Jana and Deb, 2006). The aminocyclitol core is the 2-deoxystreptamine in most aminoglycosides important for chemotherapy (Figure 2), and it can be monosubstituted in position 4, or disubstituted in positions 4 and 5, or 4 and 6. Nevertheless, there are other aminocyclitol rings that constitute the aminoglycoside structure, such as streptamine, actinamine, or fortamine. It has been extensively shown that the number and location of amino groups in the sugars attached to the ring profoundly affect the biological activity of the antibiotic (Vincens and Westhof, 2002). The presence of at least one amino group in these sugars is required for the biological activity, and the addition of extra substituents to the aminocyclitol-aminosugar (e.g. ribose moiety in the case of ribostamycin) can further increase the activity of these compounds.

More than 150 naturally-occurring aminogycosides have been isolated from Actinobacteria cultures to date, but it is the structural core that divides this group of antibiotic agents into two subgroups commonly called *typical* or *atypical* aminoglycosides. *Typical* aminoglycosides would be those consisting of a 2-deoxystreptamine as the aminocyclitol moiety and are the most commonly used for therapeutic purpose. Although we will find aminoglycosides mostly divided into 2-DOS and non-2-DOS, we may find literature classifying aminoglycosides into three structural groups based on the position of the glycosidic bonds. These groups are the 4,6-disubstituted 2-deoxystreptamines, the 4,5-disubstituted 2-deoxystreptamines, and others. It is remarkable that aminoglycosides within a certain class differ from each other in subtle ways. The main features of different aminoglycoside antibiotics are outlined in Table 1.

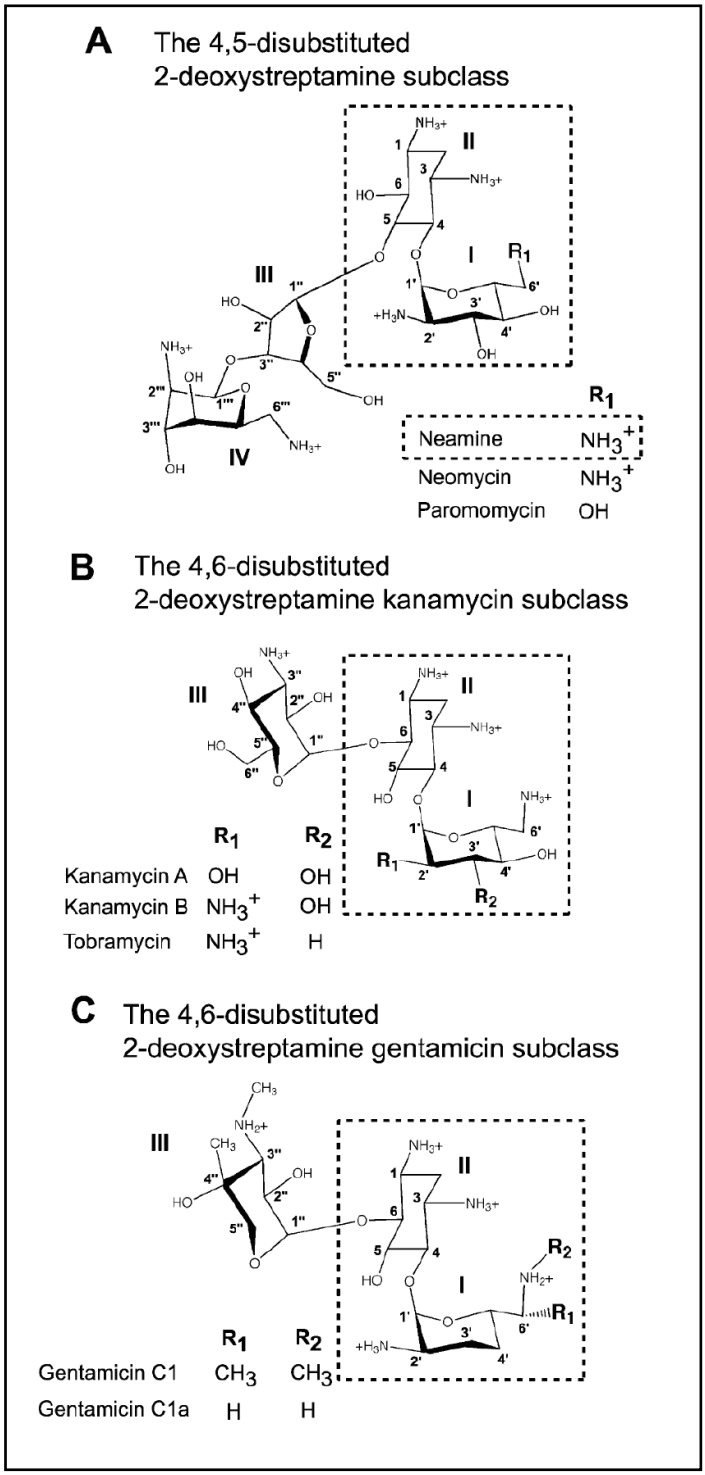

Figure 2. Chemical structure of a selection of 2-DOS aminoglycosides. Vincens and Westhof, 2002.

1.3.1.1- 2-DOS aminoglycosides

The aminoglycosides composed of a 2-DOS core can be substituted at the C4, C5, and C6 position. The 4,5-disubstituted 2-DOS are represented by the neomycins, firstly described in 1949 and produced by different members of the genus *Streptomyces.* This family includes a variety of different naturally-occurring compounds. Ribostamycin has been described as a common precursor of all other family members: neomycins, paromomycins, lividomycins, and butirosins. Some *Micromonospora* spp. have been found to produce neomycins and paromomycins. Interestingly, butirosin can be produced by species of the genus *Bacillus*.

The 4,6-disubstituted 2-DOS class contains most of the clinically useful aminoglycosides, and some of the members of this group have been used as a template for the design of new semisynthetic agents (Park *et al.*, 2013). Two families can be found in this group. The kanamycin family, firstly described in 1957 from a *S. kanamyceticus* culture, includes the naturally-produced compounds kanamycin, bekanamycin, and tobramycin; and this family has led to the creation of dibekacin, amikacin, and arbekacin as semisynthetic derivatives. The other subfamily is constituted by the gentamicins. These agents were first isolated from cultures of *M. purpurea* (which was later renamed to *M. echinospora*), in 1963. Very similar compounds were described later from cultures of other species of *Micromonospora* spp. The main naturally-produced aminoglycosides related to gentamicin are gentamicin C, gentamicin B, micronomicin, sisomicin, and geneticin. Netilmicin and isepamicin are semisynthetic derivatives that belong to this group (Magnet and Blanchard, 2005). Although the 2-DOS aminoglycosides are generally disubstituted in positions 4 and 5, or 4 and 6 as described above, there is one structurally unique aminoglycoside belonging to this group: apramycin. This compound´s aminocyclitol is also 2-deoxystreptamine but it is monosubstituted in position 4, and it is produced by various actinomycetes of the *Streptomyces* genus but also to other genera such as *Sacchalopolyspora* (Matt *et al*., 2013).

Table 1. General features of the most relevant aminoglycoside antibiotics

Aminoglycoside	Aminocyclitol	Naturally-produced (N)/Semisynthetic (S)	Origin	Discovery
Gentamicin	2-DOS (4,6-disubstituted)	N	*M. purpurea*	1963
Sisomicin		N	*M. inyoensis*	1970
Isepamicin		S	Gentamicin derivative	1974
Netilmicin		S	Sisomicin derivative	1975
Kanamycin		N	*S. kanamyceticus*	1957
Tobramycin		N	*S. tenebrarius*	1967
Dibekacin		S	Bekanamycin derivative	1971
Amikacin		S	Kanamycin derivative	1972
Arbekacin		S	Bekanamycin derivative	1973
Neomycin	2-DOS (4,5-disubstituted)	N	*S. fradiae*	1949
Apramycin	2-DOS (4-monosubstituted)	N	*S. tenebrarius*	1968
Streptomycin	streptidine	N	*S. griseus*	1944
Spectinomycin	actinamine	N	*S. spectabilis*	1961
Fortimicin	fortamine	N	*M. olivasterospora*	1977

1.3.1.2- Non 2-DOS aminoglycosides

This group represents a minority of the total of aminoglycoside compounds discovered to date, and the agents belonging to this group are not as clinically relevant as the 2-DOS aminoglycosides, with the sole exception of streptomycin. The main representative antibiotics of this group are streptomycin, spectinomycin and fortimicin (Piepersberg, 1995). Streptomycin possesses a streptidine ring as the central aminocyclitol, with a glycosidic substitution in position 4. Spectinomycin,

detected from *Streptomyces spectabilis* in 1961, does not contain an amino sugar as mentioned before. Instead, it is composed of an aminocyclitol designated actinamine, and actinospectose. Finally, fortimicins (also called astromicins), have firstly been described in cultures of *Micromonospora olivasterospora*, in 1977. Later, very similar compounds were found to be produced in other actinomycete genera and species (as is the case of dactimicin from *Dactylosporangium matsuzakiense*). The fortimicins contain an aminocyclitol called fortamine.

1.4- Therapeutic traits and applications

Aminoglycosides exhibit bactericidal activity and they can be considered as broad-spectrum antimicrobials. Only spectinomycin and kasugamycin, two inhibitors of the translocation step, act bacteriostatically. The action of this group of antibiotic agents is concentration-dependent. The 4,5- and 4,6-disubstituted 2-DOS are the most important aminoglycosides in terms of clinical therapy. Nevertheless, the growing problem of antibiotic resistance is forcing the urgent discovery or synthesis of new molecules, and for that purpose a detailed understanding of the biosynthetic pathways of aminoglycosides is crucial (Park *et al.*, 2013). Although the biosynthetic pathways of the 4,5-disubstituted 2-DOS have been elucidated (Kudo and Eguchi, 2009), those of the 4,6-disubstituted 2-DOS remain unclear, with the sole exception of the recent ascertainment of the kanamycin biosynthetic pathway (Park *et al*., 2011). This finding, combined with the engineered synthesis of an active kanamycin analogue, proves that a potential formulation of more powerful aminoglycosides is possible. Unfortunately, it is well known that certain aminoglycosides do not only display activity on the 70S ribosomes of prokaryotes, but also on the 80S ribosomes of eukaryotes, leading to significant toxicity issues and secondary effects when used, which will be further described in this chapter. Among the advantages of the aminoglycosides, we could highlight their chemical stability, as well as their rapid and broad-spectrum bactericidal action, the rarity of allergic reactions, the low cost compared to other agents, and the possibility of synergistic activity in a combined therapy with other antibiotics. On the other hand, aminoglycosides entail some disadvantages such as

the poor oral absorption, the inactivity against anaerobic bacteria, and most importantly, the different toxicities that they exhibit. The main applications of different aminoglycosides are listed in Table 2.

1.4.1- Clinical use

Most aminoglycosides exhibit a strong activity against a wide range of mainly aerobic gram-negative bacteria, including *Pseudomonas* spp., and they can also display antibactericidal action on certain mycobacteria and staphylococci (Davies, 2006). They have a poor efficacy against streptococci and they rarely inhibit the growth of anaerobic bacteria. As it has been explained before, and due to their cationic nature, aminoglycosides are poorly absorbed orally. For this reason, and in order to reach the optimal serum concentrations, as a rule they are administered parenterally (Edson and Terrel, 1999). Although during the last 20 years, aminoglycosides have been largely replaced by new generations of antibiotics such as cephalosporins, they are still widely used, remaining one of the major groups of antibiotics in chemotherapy, as stated by the World Health Organization (WHO, 2007). This family of antibiotics is especially useful to treat serious infections due to aerobic gram-negative bacteria, as well as they are a nearly ideal therapy for emergencies because of their broad spectrum and lack of allergic reactions.

The main aminoglycoside molecules available as chemotherapeutic agents are streptomycin, spectinomycin, neomycins, paromomycins, ribostamycin, kanamycin, bekanamycin, gentamicins, sisomicin, micronomicin, tobramycin, and fortimicin. In addition, five semisynthetic aminoglycosides were initially marketed as chemotherapeutic agents to combat aminoglycoside-resistant bacteria. These are netilmicin, isepamicin, amikacin, dibekacin, and arbekacin.

In current clinical practice, gentamicin, tobramycin, and amikacin are often used interchangeably as the agents of choice for the treatment of hospital acquired infections caused by *Enterobacteriaceae* and *Pseudomonas aeruginosa*. Perhaps gentamicin is the aminoglycoside most commonly used due to its low cost and

excellent activity against gram-negative aerobic bacteria. Tobramycin has shown an increased *in vitro* activity against *P. aeruginosa* in several studies, but whether this action would be significant or not *in vivo* has been questioned. The semisynthetic amikacin became an effective alternative when it was used against resistant bacteria due to modifying enzymes, as its chemical structure was developed to make it less susceptible to such modifications. Streptomycin, which was the first effective treatment for tuberculosis, is still the agent of choice to treat multi-drug resistant *Mycobacterium tuberculosis*. It is also used against tularemia (*Francisella tularensis*), and the plague (*Yersinia pestis*); and it could be an alternative treatment for brucellosis. Neomycin and paromoycin are mainly used topically for various applications, such as ulcers, wounds, or burns. The newest semisynthetic aminoglycoside, arbekacin, which is not a substrate for many aminoglycoside-modifying enzymes, became very popular in the 1990s in Japan for the treatment of infections caused by gentamicin-resistant bacteria. However, as will be further described through out this thesis, the appearance of 16S rRNA methyltransferases as a high-level resistance mechanism, led to a lack of effectiveness of most aminoglycosides including arbekacin in strains bearing a 16S rRNA methyltransferase. Nevertheless, the development of arbekacin brought an effective treatment for infections due to methicillin-resistant *S. aureus* (MRSA) (Davies, 2006).

The conventional dosing for gentamicin, tobramycin, or netilmicin is approximately 5 mg/kg per day, but some other aminoglycosides may require higher doses, as it is the case of amikacin or streptomycin. Generally, the accepted therapeutic concentration required for the inhibition of prokaryotic protein synthesis is $\leq$ 25 μg/ml for clinically used aminoglycosides. Regarding single versus multiple daily doses, it has been reported that the obtained efficacy of a single daily dose is similar, since aminoglycoside activity is concentration-dependent and effects are visible at a later stage (Freeman *et al*., 1997). This can be favourable because a single dose means less toxicity, lower cost, and reduced selection of resistant bacterial populations. In addition, it has been proved that single daily dosing is more beneficial for patients, as long as they have normal renal activity. Despite these findings, the conventionally recommended therapy

involves multiple doses per day. Further observations have shown that, although aminoglycosides are commonly administered by either an intravenous or intramuscular route, liposome-encapsulated or aerosolized aminoglycosides can be favourable against intracellular *Mycobacterium avium* or to treat respiratory tract infections. Occasionally, it is recommended to use a rotation of aminoglycosides in order to avoid treatment failures due to resistant strains.

One of the great advantages of aminoglycosides is the possibility of a combined therapy with other antibiotics that lead to a synergistic bactericidal effect due to an increased uptake of aminoglycosides by the bacterial cells. Routinely, the combination used for the treatment of serious nosocomial infections consists of gentamicin and a β-lactam, usually ampicillin. Nevertheless, other antibiotics that inhibit cell wall synthesis can be co-administered with an aminoglycoside to improve the therapy against bacteria naturally resistant to aminoglycosides due to a reduced uptake. With respect to urinary tract infections (one of the clinical targets of aminoglycosides) these antibiotics can be used without combined therapy, giving rise to a desirable efficacy.

1.4.1.1-Aminoglycosides in Veterinary Medicine

The use of aminoglycosides in the treatment of infections in animals has been moderated and it can vary depending on toxicity considerations regarding the animal treated. Furthermore, the toxicity concerns during long-term therapies have further limited the usefulness of aminoglycosides. Often, systemic use is limited to the treatment of serious gram-negative infections resistant to less toxic medication. Since the use of antibiotics as growth promoters was banned in the European Union in 2006, aminoglycosides can be employed only for clinical purposes in different animal species, including both food producing animals and companion animals.

Table 2. Main applications of aminoglycosides

Aminoglycoside	Use	
Gentamicin Amikacin Tobramycin	- hospital acquired infections - *P. aeruginosa* infections	Bactericidal in human medicine
Streptomycin	- MDR *M.tuberculosis* - tularemia - plague	
Neomycin Paromomycin	- Topical use (ulcers, burns..)	
Arbekacin	- MRSA	
Neomycin	- oral administration for enteritis in food producing animals	Bactericidal in veterinary medicine
Gentamicin Amikacin	- Respiratory/urinary tract infections and septicemias in companion animals	
Streptomycin Dihydrostreptomycin	- staphylococci - *Pasteurella* spp. - *Brucella* spp. - *Leptospira* spp. - tuberculosis	
Apramycin	- colibacillosis and salmonellosis in calves and lambs - enteritis in pigs and rabbits - septicemia in poultry	
Destomycin A Hygromycin B	- anthemintics in animals	Others
6´-OH AGs Paromomycin	- human genetic diseases	
Ribostamycin Kasugamycin	- prevention of plant diseases	

The approval of certain aminoglycosides for veterinary medicine is regulated depending on the current legislation of each country, therefore, there is a worldwide variation of the use of each aminoglycoside as an authorized antibiotic for veterinary medicine. In the same way, regulatory requirements concerning withdrawal times for food animals as well as milk discard times vary among countries. It is important to take into account, that according to the type of production and number of animals, sometimes the treatments are not individual as they are in companion animals. So when large groups of animals have to be treated, such as in poultry or swine production, the compound has to be applied via the feed or the water. This is a disadvantage for aminoglycosides due to their poor oral absorption. In general, the most commonly used aminoglycosides in veterinary medicine are gentamicin, neomycin, streptomycin and dihydrostreptomycin. Neomycin is usually administered as an oral solution for all food producing species to control enteritis caused mainly by *Escherichia coli*, whereas injections of gentamicin or amikacin are lisenced for the treatment of septicemia, respiratory and urinary tract infections, or other serious infections in animals not used for food production, such as dogs and cats. In minor species, such as parrots, ball pythons, gophers, tortoises, or guinea pigs; the safety and efficacy have not yet been established, however, both gentamicin and amikacin are recommended for the treatment of certain bacterial infections, as long as the treated animals are not used for food production. Streptomycin and dihydrostreptomycin (no longer available in the USA) are characterized by a narrow spectrum, and their efficacy is more limited by bacterial resistance. They are mainly used against staphylococci, *Pasteurella* spp., *Brucella* spp., *Leptospira* spp., and to treat tuberculosis. Although most aminoglycosides used in human medicine are authorized for veterinary medicine with exceptions depending on the animal species, there are certain aminoglycosides, such as arbekacin, restricted to human medicine. On the other hand, one aminoglycoside especially relevant for veterinary medicine, apramycin, has not been lisenced for use in humans (Matt *et al*., 2013). Apramycin has been use extensively in veterinary medicine since the early 1980s. It is considered safe in most species although it is relatively toxic in cats. Apramycin is used for the treatment of colibacillosis and salmonellosis in calves, bacterial enteritis in pigs,

colibacillosis in lambs and *E. coli* septicemia in poultry. It is also administered to rabbits, but it is not authorised for use in laying birds or in cattle or sheep producing milk for human consumption. Due to its unique structure, apramycin remained safe from the action of aminoglycoside modifying enzymes for a long time. However, the appearance of the AAC(3)-IV enzyme, which acetylates not only apramycin, but other aminoglycosides including gentamicin and tobramycin, posed a great concern as these aminoglycosides are important in human medicine.

1.4.2- Other applications of aminoglycosides

Despite the high number of aminoglycoside compounds that have been found in the last 60 years, only a few of them have been proven effective in human clinical practice as bactericidal agents, mainly those belonging to the 4,5 and 4,6-disubstituted DOS groups and streptomycin. However, some of them can be useful for other purposes. For instance, two 5-monosusbstituted DOS, called destomycin A and hygromycin B, are used as anthelmintics in animals. Furthermore, some aminoglycosides, such as paromomycin, ribostamycin and streptomycin, have shown a significant antifungal and antioomycete activity (Lee *et al.*, 2005). As these infections can be wide-host-range plant pathogens, these aminoglycosides were found to be a good weapon against significant plant diseases affecting many crops. A few aminoglycosides, such as geneticin (G-418), are available as biochemical reagents for molecular biology (Nudelman *et al*., 2010). This is due to the activity of certain aminoglycosides against the protein synthesis of eukaryotes, as the difference in binding specificity on the 70S ribosomes of prokaryotes or the 80S ribosomes of eukaryotes comes from a conserved base difference in the 16S/18S rRNA (A1408/G1408) (Eustice and Wilhem, 1984). Molecules with a hydroxyl function at C-6´ instead of an amino function, have been found to be more effective inhibitors of eukaryotic protein synthesis (Shalev *et al*., 2012). This has been exploited by several clinical studies that have been carried out over the last two decades to determine the potencial role of these aminoglycosides in the treatment of genetic diseases such as hemophilias and cystic fibrosis (James *et al*., 2005). These genetic disorders are caused by nonsense mutations that lead to the

accumulation of truncated proteins. A few aminoglycosides including G-418, gentamicin, and paromomycin have been shown to suppress premature peptide-chain termination and partially restore functional protein production in the case of more than 20 genetic diseases (Keeling and Bedwell, 2011). A temporary alleviation of the symptoms of these disorders after the administration of these aminoglycosides has been reported. However, ongoing studies are needed for further development. Nevertheless, the toxicity issues associated with these antibiotics still limit their clinical applications for this purpose.

1.4.3- Discovery of new aminoglycosides

Since the most recently approved semi-synthetic aminoglycoside arbekacin in 1990, no new aminoglycoside antibiotics have been launched. The discovery of the first aminoglycoside resistance strains via modifying enzymes led to the first investigations of the 3-D atomic structure of these enzymes as well as to the development of inhibitors of aminoglycoside resistance enzymes. The prevention of these aminoglycoside modifications is attemped via various strategies (Boehr *et al*., 2005). One strategy involves the use of aminoglycosides where the functional group that is modified by a particular enzyme is missing, for example tobramycin and dibekacin lack the 3´hydroxyl group that is phosphorylated in kanamycin by an APH(3´). Another strategy consists of modifying aminoglycosides by disrupting interactions that are critical for the affinity between the antibiotic and a particular enzyme. Also the site of inactivation can be blocked, for example with a methyl group, as in the case of the kanamycin derivative 6´-N-methylkanamycin. However, this kind of approach is not broadly applicable since hundreds of different enzymes have appearedin the last decades, and one bacterium often carries multiple enzymes. Additionally, many of the structural requirements for the bactericidal activity of aminoglycosides are targeted by resistance enzymes.

Although the modifying enzymes constitute the most widely disseminated and frequent aminoglycoside resistance mechanism, the recent emergence of the 16S rRNA methyltransferases as a high-level resistance determinant, the potential

value of certain aminoglycosides for the treatment of genetic diseases, and the toxicity issues related to the existing aminoglycosides, have led to an urgent necessity to find new aminoglycoside molecules. Over the last few years several synthetic derivatives of aminoglycosides, such as NB74 and NB84, were developed to overcome some of the toxicity issues (Nudelman *et al*., 2009). A recent medicinal chemistry campaign by the biopharmaceutical company Achaogen, yielded a collection of over 400 sisomicin analogs, and ACHN-490, later called plazomicin, was selected. ACHN-490 (plazomicin) is the only novel aminoglycoside derivative currently under clinical trial to combat MDR gram-negative pathogens, and human studies to date have not reported nephrotoxicity or ototoxicity, as well as a lack of ototoxicity has been reported in a guinea pig model (Zhanel *et al*., 2012).

1.5- Toxicity of aminoglycosides

As mentioned before, a major drawback to the use of aminoglycosides is their tendency to cause adverse effects. The side effects of this class of drugs that must be highlighted are the ototoxicity, nephrotoxicity, and in rare cases neurotoxicity (Jiang *et al*., 2006). Several factors influence the incidence of both the ototoxicity and nephrotoxicity, such as the choice of drug, duration of the treatment, or the functionality of the kidney. For instance, neomycin is more nephrotoxic and ototoxic than gentamicin or tobramycin. Longer therapies have been shown to cause a higher incidence of hearing loss, although isolated clinical cases where a single injection led to hearing loss have been reported (Forge and Schacht, 2000). This sensitivity was discovered to be associated to a mutation in the mitochondrial ribosomal RNA where a guanosine to adenosin substitution occurs at position 1555. Moreover, 17% of patients with an aminoglycoside-induced hearing loss may be carriers of this mutation. Also the physiological state of the patient is crucial, so a decreased kidney function may increase the risk of developing side effects. Other factors such as the patient age or the co-administration with other drugs have been suggested to play a role in the toxicity levels of aminoglycosides.

The mechanisms involved in aminoglycoside toxicity have been a matter of discussion. However, there is experimental evidence for aminoglycoside-induced dysfunction of mitochondrial ribosomes as a cause for the toxicity. Impaired mitochondrial function results in the formation of reactive oxygen species (ROS), which may be directly involved in the toxic effects. The interaction of aminoglycosides with metal ions, which has been called "metalloaminoglycosides", has been related to their activity and toxicity, as it enhances the formation of ROS (in metal-catalyzed oxidations) under physiological conditions. The metal-chelating abilities of aminoglycosides have been investigated in the context of cellular toxicity, and studies of iron complexes with neomycin and gentamicin have provided a molecular insight into ototoxicity pathways (Jiang *et al.*, 2007). Despite the linkage between these metal complexes and toxicity, the enhanced biological activities of the metalloaminoglycosides suggest a potential therapeutic application that is currently being investigated. Thus, a therapeutic benefit would be obtained if an appropriate metalloaminoglycoside could damage the target molecules, while promoting a beneficial chemistry and minimizing the toxic side effects. The general symptoms observed after aminoglycoside-induced toxicity are summarized in Figure 3.

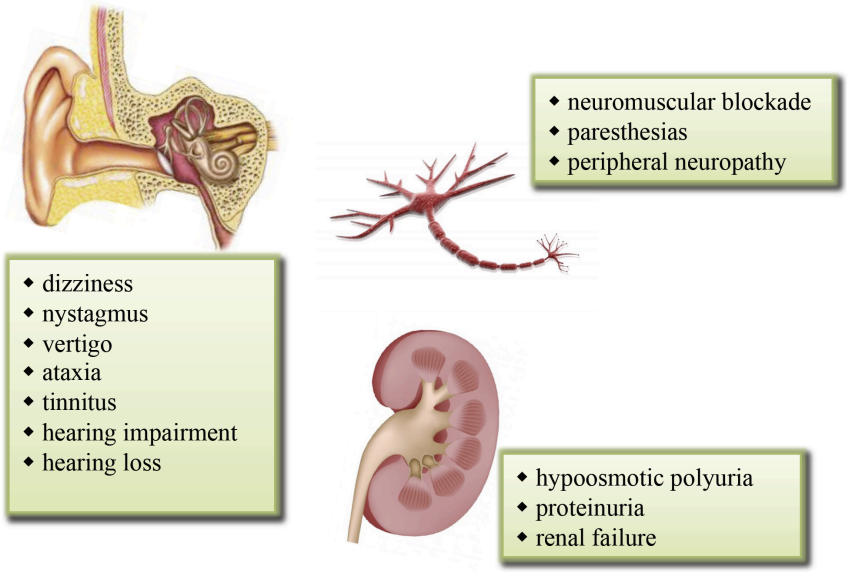

Figure 3. General effects of aminoglycoside-induced toxicity.

1.5.1- Ototoxicity

This toxicity is considered the major disadvantage of aminoglycosides, as, in contrast to the renal effects, which can be reverted, the ear damages are irreversible, leading to permanent loss of hearing and balance. Ototoxicity has been associated with both natural and semisynthetic aminoglycosides (Edson and Terrel, 1999). The ototoxicity is associated with the destruction of the sensory cells of the inner ear, which are essential for the transduction of auditory stimuli and the sensation of balance. Aminoglycosides enter the inner ear through the bloodstream rapidly after parenteral administration. The concentration of the drug reached in the inner ear does not necessarily correlate with the severity of damage, however its extreme persistence does, since despite their quick tissue penetration, aminoglycosides are not efficiently cleared. Thus, whereas the half-life of most aminoglycosides in the blood is around 3-5 hours, their half-life in the inner ear tissues may be up to 30 days. Ototoxic effects usually develop after the completion of the treatment. These effects include damage to the vestibular system, which results in balance disorders, and damage to the cochlea, resulting in hearing loss. Aminoglycosides can cause one or more of these effects, although the tissue targets can vary and this variation does not follow a structure-activity relationship. In other words, gentamicin is more vestibulotoxic than cochleotoxic in humans whereas amikacin or neomycin are more cochleotoxic. On the other hand, streptomycin is considered exclusively vestibulotoxic. Likewise, there is no correlation between the effects of certain aminoglycoside on the inner ear and on the kidney. Interestingly, the ototoxicity of gentamicin can be exploited to treat some individuals with Ménière's disease by destroying the inner ear, which stops the vertigo attacks but causes permanent deafness.

1.5.2- Nephrotoxicity

As a consequence of the aminoglycosides' polar structure, they poorly cross membranes and thus the intracellular tissue concentration is low except in the proximal renal tubule where this concentration is high. Approximately, 5% of the

administered dose is retained in the epithelial cells lining the S1 and S2 segments of the proximal tubules after glomerular filtration. After several days of administering the clinical dose, signs of tubular dysfunctions may appear. In humans, these signs may be followed by an overt renal failure. However, a recovery after the termination of the treatment is often observed. In contrast, in animals, higher doses (i.e. 40mg/kg or more for gentamicin) are necessary to induce an overt renal dysfunction after the first symptoms (Mingeot-Leclercq and Tulkens, 1999). Once-a-day dosing is a successful strategy to avoid nephrotoxicity, although other protective approaches such as the co-administration of polyaspartic acid or deferroxamine have also been shown to diminish the nephrotoxic effects.

CHAPTER 2. Bacterial resistance to aminoglycosides

Despite the significant improvement of medicine when antibiotics became available for the treatment of bacterial infections, the situation drastically changed as soon as bacteria responded by manifesting resistance to those antibiotics. Nowadays, antibiotic resistance poses one of the major threats for Public Health, as it often results in treatment failure, which has serious consequences especially in critically ill patients (Tenover, 2006). Furthermore, the continuous and rapid emergence and spread of resistance mechanisms to all clinically available antibiotics compared to the lack of discovery of new bactericidal molecules in the last decades poses a great concern regarding the future of infection control. This situation has been aggravated due to the misuse of antibiotics, which is a contributing factor for the selection of resistant bacteria, and in the last years, several Governments and Public Institutions worldwide have raised awareness concerning the correct use of antibiotics through various campaigns (Huttner *et al*., 2010) (Figure 4). This problem is not only related to nosocomial infections within healthcare institutions, but to community-acquired infections as well. Moreover, antibacterial drug resistance adds a burden to the healthcare systems (McGowan, 2001).

Usually, susceptible bacteria may become resistant to antibiotics through mutation, or by acquiring the genetic information that encodes resistance from other bacteria (genetic exchange). The mechanisms of genetic exchange in bacteria include conjugation, transformation, and transduction, which accelerate the spread of resistance via horizontal gene transfer that may occur between strains from the same or different species or genera (McManus, 1997). This type of acquired resistance seriously contributes to the appearance of multidrug resistant bacteria that have become a cause of major concern, particularly in hospitals.

Figure 4. Campaigns that promote the rational use of antibiotics.

Aminoglycosides were one of the first antibiotic classes identified during the "Golden Age" of antibiotic discovery (late 1940s-early 1960s), and they were seen a clinical revolution due to their broad-spectrum and excellent bactericidal activity against insidious gram-negative bacteria and mycobacteria (Falagas *et al.*, 2011). In line with other antibiotics, resistance to aminoglycosides has had a profound impact on the usefulness of these agents. The first reports of enzyme-mediated aminoglycoside resistance by Umezawa and colleagues in the 1960s, spurred the search for new aminoglycosides. The natural compound tobramycin and the semisynthetic compound amikacin, introduced in the early 1970s, avoided the first emerged resistance mechanisms to gentamicin and kanamycin, but since then, only a few aminoglycosides were launched into clinic use, mostly in Japan. The most propitious of those were isepamicin and arbekacin. The general mechanisms of bacterial resistance to aminoglycosides are the reduction of the intracellular concentration of the antibiotic, the enzymatic modification of the drug,

and the modification of the molecular target. These mechanisms will be extensively described in chapters 2 and 3.

2.1- Decreased intracellular concentration

The reduced concentration of aminoglycosides inside the bacterial cell is due to an altered transport via import or export mechanisms. Thus, the lower antibiotic concentration is achieved either by a reduction of drug uptake or an expulsion of the drug by efflux systems.

2.1.1- Reduced aminoglycoside uptake

Aminoglycoside entry to the cell has been shown to require a proton motive force generated by the respiratory chain. Therefore, anaerobic bacteria are intrinsically resistant to aminoglycosides due to the lack of drug uptake. In the same manner, mutants deficient in components of the electron transport chain, such as ATP synthases, show an altered aminoglycosides transport and are consequently resistant. This type of mutant has been isolated from endocardium infections caused by *E. coli*, *P. aeruginosa*, and *S. aureus* (Balwit *et al.*, 1994). In *P. aeruginosa* it is more frequent than in other bacterial species to observed a reduced susceptibility to aminoglycosides due to a diminished uptake. Gentamicin resistance conferred by the inactivation of OprH, an outer-membrane porin protein, has been reported. The implication of the two-component regulatory system *phoP-phoQ* on this resistance has also been shown, as these are the regulators of several genes involved in lipopolysaccharide modification (Bryan *et al.*, 1984). When the lipopolysaccharide is less negatively charged, it exhibits a lower affinity for gentamicin. Furthermore, the alginate gel excreted by mucoid strains of *P. aeruginosa*, seems to play a role in the decreased aminoglycoside uptake, likely due an ionic trap for the antibiotic molecule (Hatch and Schiller, 1998).

2.1.2- Aminoglycoside efflux

Antibiotic efflux is an energy-dependent process that significantly contributes to multidrug resistance in a number of bacteria. In the last years, various efflux mechanisms whose genes are located on either plasmids or chromosomes have been described in a variety of organisms. Some of these mechanisms are agent- or class-specific while others are non-specific. There are five families of transmembrane efflux systems associated with antibiotic resistance (Table 3) (Poole, 2005). These are the major facilitator superfamily (MF), the ATP-binding cassette family (ABC), the resistance-nodulation division family (RND), the small multidrug resistance family (SMR), and the multidrug and toxic compound extrusion family (MATE). Usually, bacteria constitutively expressing these systems are intrinsically resistant to low levels of various antibiotics. However, when there are mutations in the regulatory genes of the pumps, or an induction of the expression in the presence of the substrate, there is an overexpression of the pump genes that may lead to an increased resistance. The efflux system most relevant to gram-negative resistant bacteria is the resistance-nodulation division family (RND). The transporters of this family use the membrane proton motive force as an energy source. An RND system consists of three major components: the RND pump, a periplasmic membrane fusion protein (MFP), and an outer-membrane factor (OMF) (Figure 5). In recent years, aminoglycosides have been shown to act as substrates for a number of multidrug efflux pumps belonging to the five families, although the majority of known aminoglycoside exporters belong to the RND family (Poole, 2005). Nevertheless,

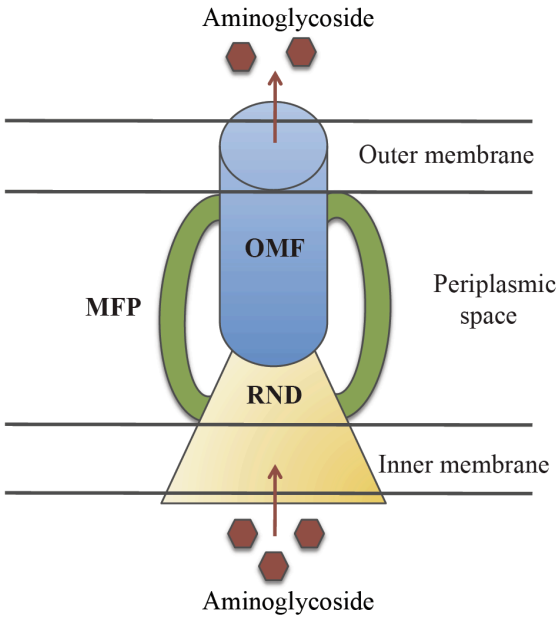

Figure 5. A representative RND efflux system consisting of three main components: OMF (outer membrane factor), MFP (membrane fusion protein), RND (pump).

most RND pumps act more effectively against lipophilic and amphiphilic substrates, thus, the hydrophilic aminoglycoside molecules are poor substrates for these transporters. Aminoglycoside efflux is a significant mechanism of conferring aminoglycoside resistance in bacteria of the genera *Pseudomonas*, *Burkholderia*, and *Stenotrophomonas*, although efflux-mediated aminoglycoside resistance has also been described in other bacteria. The first aminoglycoside efflux system, AmrAB-OprA, was reported in *Burkholderia pseudomalei*. Since then, others have been reported in *P. aeruginosa, E. coli*, *Lactococcus lactis, Acinetobacter baumanii,* and *Stenotrophomonas* spp. (Table 3). The first pump found in *P. aeruginosa* was the MexAB-OprM system, although its ability to expel aminoglycosides is restricted to low-ionic-strength media (Masuda *et al.*, 2000). The most effective efflux pump in *P. aeruginosa* is MexXY-OprM (Aires *et al.*, 1999). The genes *mexXY* are co-located in an operon with *mexZ*, which encodes for the negative regulator of *mexXY*. The expression of *mexXY* is inducible by aminoglycosides, so the exposure of the bacterium to any aminoglycoside leads to an enhanced pan-aminoglycoside resistance that is, however, quickly lost in the absence of the drug.

Table 3. Families of efflux systems associated with antibiotic resistance, and main pumps involved in resistance to aminoglycosides.

Family	Efflux system	Organism	Location
MF	MdfA	*E. coli*	Plasmid
ABC	LmrA	*L. lactis*	Chromosome
	AmrAB-OprB	*B. pseudomallei*	Chromosome
	BpeAB-OprB	*B. pseudomallei*	Chromosome
	MexAB-OprM	*P. aeruginosa*	Chromosome
RND	MexXY-OprM	*P. aeruginosa*	Chromosome
	AcrAD-TolC	*E. coli*	Chromosome
	AdeAB-AdeC	*A. baumanii*	Chromosome
	SmeAB-SmeC	*S. maltophilia*	Chromosome
SMR	EmrE	*P. aeruginosa*	Chromosome
MATE	AbeM	*A. baumanii*	Chromosome

In *E. coli*, AcrAD-TolC is the aminoglycoside resistance efflux-mediated system homologous to MexXY-OprM. There are several AcrD homologs in other members of the *Enterobacteriaceae* family, which suggests that this pan-aminoglycoside resistance system may exist in other gram-negative bacteria. Although the efflux genes involved in aminoglycoside resistance are located in the bacterial chromosome, members of the major facilitator superfamily (MF) have also been shown to decrease aminoglycoside susceptibility in strains harbouring the structural gene on a multicopy plasmid.

2.2- Enzymatic drug modification

The aminoglycoside modifying enzymes are the most prevalent mechanism of aminoglycoside resistance (Tolmasky, 2007). They are classified into three families: the aminoglycoside *N*-acetyltransferases (AACs), the aminoglycoside *O*-nucleotidyltransferases (ANTs), and the aminoglycoside *O*-phosphotransferases (APHs). These enzymes modify their target aminoglycosides at −OH or $-NH_2$ groups by using either ATP or acetylCoA as co-substrates. The modified groups of the antibiotic are usually essential for aminoglycoside binding to the ribosome (Figure 6). Many of the genes encoding these enzymes are located on mobile genetic elements facilitating horizontal gene transfer. Nevertheless, some bacteria also bear these genes in the chromosome, for example, the *Enterococcus faecium* chromosome harbours a ubiquitous gene encoding for the AAC(6´)-Ii enzyme. Due to the high number of enzymes, their rapid spread, and their continuous evolution, this mechanism is widespread among all clinically important bacterial species (Shaw *et al.*, 1993).

The large number of aminoglycoside modifying enzymes and their encoding genes has required a standardized nomenclature. However, there are still two main nomenclatures currently in use for these enzymes (Novick *et al.*, 1976; Vanhoof *et al.*, 1998). The oldest nomenclature system designates the genes as *aac* (for AACs), *aad* (for ANTs), and *aph* (for APHs) followed by a capital letter that identifies the modification site in the aminoglycoside (e.g. *aacA* stands for

aminoglycoside 6′-*N*- acetyltransferase). Subsequently a number that identifies a specific gene is added (i.e., *aacA3*). The other nomenclature system consists of AAC, ANT, or APH, referring to the family and related to the type of activity, followed by the modification site in parenthesis (class), a roman numeral referring to the resistance phenotype (subclass), and a lower case letter for the specific gene (i.e., AAC(6′)-Ia). Authors differentially prefer one system or the other, as both of them have advantages and disadvantages. But often, there is confusion or duplication when naming new variants, making it difficult to follow the updates in this field. For this reason it has already been suggested to use a single nomenclature system, which will facilitate the classification and study of these enzymes.

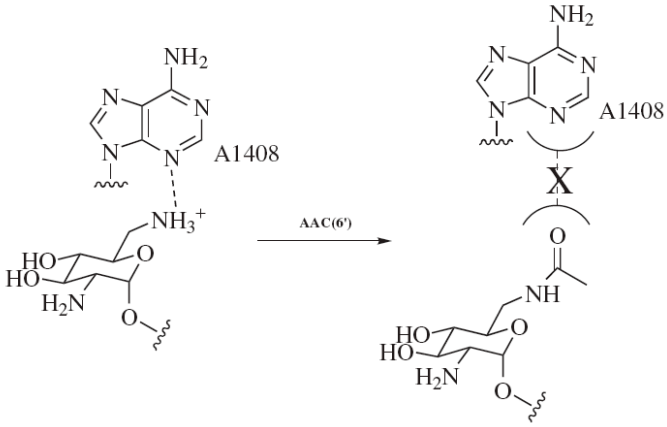

Figure 6. Modification of the 6′-NH_2 of the 2-DOS which binds to the A1408 of the bacterial 16S rRNA.

2.2.1- Aminoglycoside N-acetyltransferases (AACs)

The AACs catalyze the acetyl-CoA-dependent N-acetylation of one of the four amino groups of the typical aminoglycosides. This acetylation reduces the affinity of these compounds for their target in the 30S ribosomal subunit. The acetylation occurs after the binding of acetyl-CoA and the amino group of the antibiotic takes place, and Levings and colleagues proposed a direct nucleophilic

attack by the amine on the thioester (Levings *et al.*, 2005). Enzymes belonging to this family are present in both gram-positive and gram-negative bacteria, and they generally show a very broad aminoglycoside resistance profile.

The 3D structures of various acetyltransferases have been resolved, showing that they share similarity to one another, but they are also linked to the GCN5-related *N*-acetyltransferases (GNAT) superfamily (Azucena and Mobashery, 2001). The GNAT family is characterized by low amino acid sequence similarity but share a common 3D structure. Thus, even though the amino acid sequences of the AACs are quite different from one another, they present similar 3D-folding patterns formed around the acetyl-CoA binding pocket. The characteristic motifs that constitute the structure of these enzymes are: an N-terminal α-helix, a central antiparallel β-sheet, and a four-stranded mixed β-sheet flanked by two α-helices at the C-terminus (Figure 7).

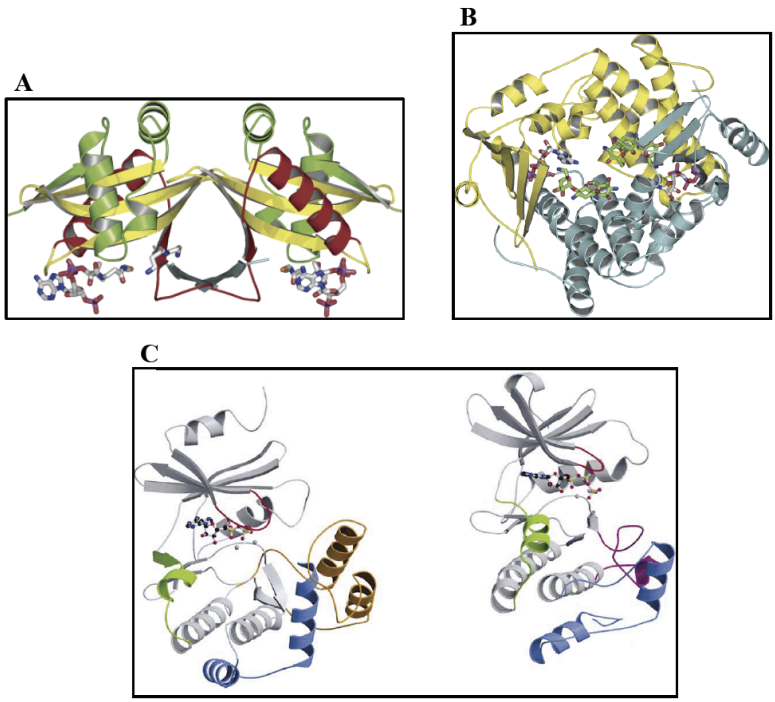

Figure 7. The 3D structure of some aminoglycoside modifying enzymes. **A:** AAC(3)-CoA complex; **B:** ANT(4´)-kanamycin A complex; **C:** Comparison of the APH(3´)-IIIa and residues 35-280 of the catalytic subunit of a eukaryotic protein kinase. A and B adapted from Magnet and Blanchard, 2005. C adapted from Burk *et al.*, 2002.

The GNAT enzymes are able to modify different acyl-accepting substrates, thus, AACs have been shown to modify not only aminoglycosides but other substrates (i.e., AAC(6′)-Ii acetylates certain GNAT substrates such as ribonuclease A or histones). Furthermore, aminoglycoside binding to the AACs may require the presence of water molecules. These indicators have led to the hypothesis that AACs may have other functions in the cell and could have evolved the ability to modify aminoglycosides (Houghton *et al.*, 2010). This family of enzymes is continuously evolving, evidence of that being the discovery of the bifunctional AAC(6′)-Ib-cr a few years ago, which is a variant of AAC(6′)-Ib that is able to also modify fluoroquinolones (Vetting *et al.*, 2008).

The AAC family are classified into four major classes based on their regiospecificity of acetyl transfer on the aminoglycoside structure: AAC(6′), AAC(2′), AAC(1), and AAC(3). The aminoglycoside *N*-acetyltransferases are listed in Table 4.

2.2.1.1- AAC(6′)

As it is extensively known, the 6′-amino group of aminoglycosides plays an important role in their binding to the 30S ribosomal subunit and the subsequent antibacterial activity. Therefore, it is not surprising that this group is targetted by a major class of aminoglycoside-modifying enzymes, the AAC(6′) class (Ramirez and Tolmasky, 2010). These enzymes are the most common and they can be extensively found in both gram-positives and gram-negatives, on plasmids and chromosomes. There are two main subclasses of AAC(6′) enzymes depending on their resistance profiles, AAC(6′)-I and AAC(6′)-II. The subclass AAC(6′)-I exhibits resistance to gentamicin C1a and C2, and to amikacin, but not to gentamicin C1, whereas the AAC(6′)-II enzymes show resistance to the three gentamicins but not to amikacin. A derivative of AAC(6′)-Ib with two amino acids, AAC(6′)-Ib-cr, shows additional resistance to fluoroquinolones and it is considered a third subclass of AAC(6′) enzymes. These three subclasses were thought to be very related, however, Salipante and Hall have suggested that they are less related

and the 6´acetylating activity has evolved independently at least three times (Salipante and Hall, 2003). Due to the large number of genes identified belonging to this class of enzymes, plus the absence of a unique nomenclature system, there have been several mistakes and difficulties when naming and classifying AAC(6´) enzymes. The existence of fusion proteins containing AAC(6´) activities has been reported, being the AAC(6´) protein located on the N or C terminal region of the composite protein. An AAC(6´) has been found fused to an APH, an ANT, AAC(6´), and to other AAC enzymes (Zhang *et al.*, 2009). For instance, the AAC(6´)-APH(2´´) is a bifunctional enzyme found in *Enterococcus faecalis* and *S. aureus*, and the gene is usually found in Tn4001-like transposons (Culebras and Martinez, 1999).

One of the most clinically relevant acetyltransferases is AAC(6´)-Ib, isolated from *Acinetobacter*, *Enterobacteriaceae*, *Pseudomonadaceae*, and *Vibrionaceae*. Its gene is mainly found within class 1 integrons, although the genetic environment is particular. Instead of the normal *attI* recombination site adjacent to the *intI* gene upstream of the *aac(6´)-Ib* gene, there is an 8 bp sequence known as *attI1** near the beginning of the gene (Centron and Roy, 2002). Many variants of this enzyme that differ in their N-terminal domains have been found, and this may be due to the high mobility of these genes (Casin *et al.*, 1998). There is a high flexibility in the structural requirements of the protein N-terminal, as most of these variants are active, this may also contribute to the successful predominance of this protein among *Enterobacteriaceae.* Some of these variants show an extended spectrum. For instance, variant AAC(6´)-Ib_{11} confers resistance to all three gentamicins, and AAC(6´)-Ib-cr leads to a reduced susceptibility to quinolones, and it has been frequently found in different integrons associated with quinolone resistance genes such as *qnr* or *qepA*, and β-lactamase genes.

2.2.1.2- AAC(1)

This class of acetyltransferase is not very frequent compared to the AAC(6´) enzymes, and its importance is minor because they do not modify the

most clinically relevant aminoglycosides. They have been found in actinomycete, *E. coli*, and *Campylobacter* spp. (Lovering *et al.*, 1987; Sunada *et al.*, 1999). The substrate profile of AAC(1) enzymes consists of neomycin, ribostamycin, apramycin, butirosin, paromomycin and lividomycin. Whereas the substrate profile of the AAC(1) isolated from *E. coli* and *Campylobacter* spp. is similar, the AAC(1) isolated from an actinomycete is not able not acetylate apramycin.

2.2.1.3- AAC(2′)

There are five aminoglycoside acetyltransferases that catalyze the acetylation of the 2′- amino group, and only one subclass exists. AAC(2′)-Ia was the first one identified from *Providencia stuartii*, while AAC(2′)-Ib, AAC(2′)-Ic, AAC(2′)-Id, and AAC(2′)-Ie have been identified in different *Mycobacterium* spp. (Ainsa *et al.*, 1997; Hegde *et al.*, 2001; Rather *et al.*, 1993). AAC(2′)-Ib has also been isolated from *A. baumanii*. These five enzymes are chromosomally encoded and are generally species specific, they are also considered to be universally present in mycobacteria. These modify various aminoglycosides including gentamicin, kanamycin, tobramycin, netilmicin, and dibekacin. Regarding AAC(2′)-Ia, a low level of expression of the gene that is not able to confer aminoglycoside resistance has been reported in wild-type bacteria, however, this gene's regulation is complex. Some studies have shown that a mutation in the *aac(2′)-Ia* gene leads to differences in cell morphology due to altered levels of peptidoglycan *O*-acetylation, thus, this may be a physiologic function of the AAC(2′)-Ia enzyme.

The three-dimensional structure of the AAC(2′)-Ic has been reported, confirming that it belongs to the Gcn5-related *N*-acetyltransferase (GNAT) superfamily. The activity of this enzyme is highest with aminoglycosides containing a 2′-amino group, however, in contrast with other acetyltransferases, AAC(2′)-Ic is active against kanamycin A and amikacin, which contain a 2′-hidroxyl group. Therefore, this enzyme may catalyze *O*-acetylation.

2.2.1.4- AAC(3)

This family is one of the largest and includes nine subclasses described to date based on the pattern of aminoglycoside resistance that they confer (Shaw *et al.*, 1993). The subclasses are named AAC(3)-I to AAC(3)-X except subclass AAC(3)-V, which was eliminated when it was shown that the only enzyme belonging to this group was the previously described AAC(3)-II.

Five enzymes have been identified within the subclass AAC(3)-I (a-e) widely distributed among gram-negative bacteria and they are characterized by conferring resistance to fortimicin, sisomicin and gentamicin. The AAC(3)-Ia originally identified in 1991 from *Serratia marcescens* was the first aminoglycoside acetyltransferase whose three-dimensional structure complexed to CoA was determined showing that the monomer fold was typical of the GNAT superfamily. The genes encoding these five enzymes have been found within integron cassettes.

The subclass AAC(3)-II includes three enzymes, and they confer resistance to gentamicin, tobramycin, sisomicin, netilmicin, and dibekacin. AAC(3)-IIa has been found widely distributed among different genera, while AAC(3)-IIb has only been identified in *E. coli*, *Alcaligenes faecalis* and *S. marcescens*, and AAC(3)-IIc in *P. aeruginosa* and *E. coli*.

As with the subclass AAC(3)-II, the subclass AAC(3)-III is comprised of three enzymes, all of them have been isolated from *P. aeruginosa*. When the *aac(3)-IIIa* gene was firstly cloned, it was expressed in *P. aeruginosa* but not in *E. coli*, which was explained by the authors as the incomplete synthesis of the mRNA or an obstruction of the initiation of translation (Vliegenthart *et al.*, 1991).

Table 4. Aminoglycoside *N*-acetyltransferases

AAC	Gene names	Host	Genetic location
AAC(1)	*aac(1)*	*E. coli*, Actinomycetales, *Campylobacter* spp.	ND
AAC(3)-Ia*	*aac(3)-Ia, aacC1*	*S. marcescens, E. coli, A. baumanii, K. pneumoniae, K. oxytoca, P. aeruginosa, S. typhimurium, P. mirabilis*	Plasmid, transposon, integron
AAC(3)-Ib	*aac(3)-Ib*	*P. aeruginosa*	Integron
AAC(3)-Ic	*aac(3)-Ic*	*P. aeruginosa*	Integron
AAC(3)-Id	*aac(3)-Id*	*S. enterica, P. mirabilis, Vibrio fluvialis*	Genomic island, integron
AAC(3)-Ie	*aac(3)-Ie, aacCA5*	*S. enterica, P. mirabilis, P. aeruginosa*	Integron
AAC(3)-IIa	*aac(3)-IIa, aacC3, aacC5, aacC2, aac(3)-Va*	*K. pneumoniae, E. cloacae, Actinobacillus pleuropneumoniae, S.* Typhimurium, *C. freundii*	Plasmid
AAC(3)-IIb	*aac(3)-IIb, aac(3)-Vb*	*E. coli, E. faecalis, S. marcescens*	ND
AAC(3)-IIc	*aac(3)-IIc, aacC2*	*E. coli, P. aeruginosa*	Plasmid
AAC(3)-IIIa	*aac(3)-IIIa, aacC3*	*P. aeruginosa*	Chromosome
AAC(3)-IIIb	*aac(3)-IIIb*	*P. aeruginosa*	ND
AAC(3)-IIIc	*aac(3)-IIIc*	*P. aeruginosa*	ND
AAC(3)-IVa	*aac(3)-IVa*	*E. coli, C. jejuni, P. stutzeri*	Plasmid
AAC(3)-VIa	*aac(3)-VIa*	*E. cloacae, S. enterica, E. coli*	Plasmid
AAC(3)-VIIa	*aac(3)-VIIa, aacC7*	*Streptomyces rimosus*	Chromosome
AAC(3)-VIIIa	*aac(3)-VIIIa, aacC8*	*Streptomyces fradiae*	Chromosome
AAC(3)-IXa	*aac(3)-IXa, aacC9*	*Micromonospora chalcea*	Chromosome
AAC(3)-X	*aac(3)-Xa*	*Streptomyces griseus*	Chromosome
AAC(2′)-Ia	*aac(2′)-Ia*	*P. stuartii*	Chromosome
AAC(2′)-Ib	*aac(2′)-Ib*	*M. fortuitum, A. baumanii*	Chromosome
AAC(2′)-Ic*	*aac(2′)-Ic*	*M. tuberculosis, M. bovis*	Chromosome
AAC(2′)-Id	*aac(2′)-Id*	*M. smegmatis*	Chromosome
AAC(2′)-Ie	*aac(2′)-Ie*	*M. leprae*	Chromosome
AAC(6′)-Ia	*aac(6′)-Ia, aacA1*	*Citrobacter diversus, E. coli, K. pneumoniae, Shigella sonnei*	Plasmid, transposon, integron
AAC(6′)-Ib*	*aac(6′)-Ib, aac(6′)-4, aacA4*	*K. pneumoniae, P. mirabilis, P. aeruginosa, S. enterica, K. oxytoca, S. maltophilia, E. cloacae*	Plasmid, transposon, integron

AAC(6´)-Ib'	*aac(6´)-Ib', aac(6´)-Ib$_6$*	*P. fluorescens, P. aeruginosa*	Integron
AAC(6´)-Ic	*aac(6´)-Ic*	*S. marcescens*	Chromosome
AAC(6´)-Ie	*aac(6´)-Ie, aac(6´)-* bifuncional	*S. aureus, Macrococcus caseolyticus, E. faecalis, E. faecium*	Transposon
AAC(6´)-If	*aac(6´)-If*	*E. cloacae*	Plasmid
AAC(6´)-Ig	*aac(6´)-Ig*	*Acinetobacter haemolyticus*	Chromosome
AAC(6´)-Ih	*aac(6´)-Ih*	*A. baumanii*	Plasmid
AAC(6´)-Ii*	*aac(6´)-Ii*	*Enterococcus* spp.	Chromosome
AAC(6´)-Ij	*aac(6´)-Ij*	*Acinetobacter genomosp. 13*	Chromosome
AAC(6´)-Ik	*aac(6´)-Ik*	*Acinetobacter* spp.	Chromosome
AAC(6´)-Ip	*aac(6´)-Ip, aac(6´)-Il, aac(6´)-Im*	*C. freundii*	Integron
AAC(6´)-Iq	*aac(6´)-Iq*	*K. pneumoniae*	Plasmid, integron
AAC(6´)-Im	*aac(6´)-Im*	*E. coli, E. faecium*	Plasmid
AAC(6´)-Il	*aac(6´)-Il, aacA7*	*Enterobacter aerogenes*	Plasmid, integron
AAC(6´)-Ir	*aac(6´)-Ir*	*A. genomosp. 14*	Chromosome
AAC(6´)-Is	*aac(6´)-Is*	*A. genomosp. 15*	Chromosome
AAC(6´)-Isa	*aac(6´)-Isa*	*Streptomyces albulus*	Plasmid
AAC(6´)-It	*aac(6´)-It*	*A. genomosp. 16*	Chromosome
AAC(6´)-Iu	*aac(6´)-Iu*	*A. genomosp. 17*	Chromosome
AAC(6´)-Iv	*aac(6´)-Iv*	*Acinetobacter* sp.	Chromosome
AAC(6´)-Iw	*aac(6´)-Iw*	*Acinetobacter* sp.	Chromosome
AAC(6´)-Ix	*aac(6´)-Ix*	*Acinetobacter* sp.	Chromosome
AAC(6´)-Iy*	*aac(6´)-Iy*	*S. enteritidis, S. enterica*	Chromosome
AAC(6´)-Iz	*aac(6´)-Iz*	*S. maltophilia*	Chromosome
AAC(6´)-Iaa	*aac(6´)-Iaa*	*S.* Typhimurium	Chromosome
AAC(6´)-Iad	*aac(6´)-Iad*	*A. genomosp. 3*	Plasmid
AAC(6´)-Iae	*aac(6´)-Iae*	*P. aeruginosa, S. enterica*	Ingegron
AAC(6´)-Iaf	*aac(6´)-Iaf*	*P. aeruginosa*	Plasmid, integron
AAC(6´)-Iai	*aac(6´)-Iai*	*P. aeruginosa*	Plasmid, integron
AAC(6´)-Ib$_3$	*aac(6´)-Ib$_3$, aac(6´)-Ib$_5$*	*P. aeruginosa*	Integron
AAC(6´)-Ib$_4$	*aac(6´)-Ib$_4$*	*Serratia* spp.	ND
AAC(6´)-Ib$_7$	*aac(6´)-Ib$_7$*	*E. cloacae, C. freundii*	Plasmid

AAC(6´)-Ib$_8$	*aac(6´)-Ib$_8$*	*E. cloacae*	Plasmid
AAC(6´)-Ib$_9$	*aac(6´)-Ib$_9$*	*P. aeruginosa*	Integron
AAC(6´)-Ib$_{10}$	*aac(6´)-Ib$_{10}$*	*P. aeruginosa*	ND
AAC(6´)-Ib$_{11}$*	*aac(6´)-Ib$_{11}$*	*S. enterica*	Integron
AAC(6´)-29a	*aac(6´)-29a*	*P. aeruginosa*	Integron
AAC(6´)-29b	*aac(6´)-29b*	*P. aeruginosa*	Integron
AAC(6´)-31	*aac(6´)-31*	*Pseudomonas putida, A. baumannii, K. pneumoniae*	Integron
AAC(6´)-32	*aac(6´)-32*	*P. aeruginosa*	Plasmid, integron
AAC(6´)-33	*aac(6´)-33*	*P. aeruginosa*	Integron
AAC(6´)-I30	*aac(6´)-I30*	*S. enterica*	Integron
AAC(6´)-Iid	*aac(6´)-Iid*	*Enterococcus durans*	Chromosome
AAC(6´)-Iih	*aac(6´)-Iih*	*Enterococcus hirae*	Chromosome
AAC(6´)-Ib-Suzhou	*aac(6´)-Ib-Suzhou*	*E. cloacae, K. pneumoniae*	ND
AAC(6´)-Ib-Hangzhou	*aac(6´)-Ib-Hangzhou*	*A. baumannii*	ND
AAC(6´)-SK	*aac(6´)-sk*	*Streptomyces kanamyceticus*	Chromosome
AAC(6´)-IIa	*aac(6´)-IIa*	*P. aeruginosa, S. enterica*	Plasmid, integron
AAC(6´)-IIb	*aac(6´)-Iib*	*P. fluorescens*	Integron
AAC(6´)-IIc	*aac(6´)-IIc*	*E. cloacae*	Plasmid, integron
AAC(6´)-Ib-cr	*aac(6´)-Ib-cr*	*Enterobacteriaceae*	Plasmid, transposon, integron
AAC(6´)-Ie-APH(2")-Ia	*aac(6´)-Ie-aph(2")-Ia*	*S. aureus, E. faecalis, E. faecium, Staphylococcus warneri*	Plasmid, transposon
ANT(3")-Ii-AAC(6´)-IId	*ant(3")-Ii-aac(6´)-IId, ant(3")-Ih-aac(6´)-IId*	*S. marcescens*	Integron
AAC(6´)-30/AAC(6´)-Ib'	*aac(6´)-30/aac(6´)-Ib'*	*P. aeruginosa*	Integron
AAC(3)-Ib/AAC(6´)-Ib"	*aac(3)-Ib/aac(6´)-Ib"*	*P. aeruginosa*	Integron

* Those enzymes for which the three dimensional structure has been resolved. Adapted from Ramirez and Tolmasky, 2010.

Within the subclasses IV and VI, a sole enzyme has been described. AAC(3)-IVa has been identified in clinical *E. coli* and *Campylobacter jejuni,* as well as environmental *Pseudomonas stutzeri*. In the case of AAC(3)-VIa, when the

original amino acid sequence from *Enterobacter cloacae* is compared to those later identified in *E. coli* and *S. enterica*, it shows one amino acid change.

Finally, the subclasses VII, VIII, IX, and X, have been identified among different strains of actinomycetes. Interestingly, AAC(3)-X is the only described AAC with AAC(3") activity, as it is able to acetylate not only the 3-amino group of kanamycin and dibekacin but the 3"-amino group of amikacin and arbekacin (Hotta *et al.*, 1998). However, the latter remains active after this modification.

2.2.2- Aminoglycoside O-phosphotransferases (APHs)

These enzymes catalyze the ATP-dependent phosphorylation of key hydroxyl substituents present on the aminoglycoside molecule. They are widely distributed among bacterial pathogens and are encoded by genes usually found on multidrug resistance plasmids and transposons.

Table 5. List of the existing APHs, and the classes and subclasses that they belong to.

Class	Subclass	Members*
APH(4)	I	Ia (*hph*), Ib (*hyg*)
APH(6)	I	Ia (*aphD*, *strA*), Ib (*sph*), Ic, Id (*strB*, *orfI*)
APH(9)	I	Ia, Ib (*spcN*)
APH(3´)	I, II, III, IV, V, VI, VII	Ia (*aphA-1*), Ib (*aphA*), Ic (*aphA7*, *aphA1-Iab*), IIa (*aphA-2*), IIb, IIc, IIIa, IVa, Va, Vb, Vc, VIa (*aphA-6*), VIb, VIIa (*aphA-7*)
APH(2")	I, II, III, IV	Ia, Ie, IIa (Ib), IIIa (Ic), IVa (Id)
APH(3")	I	Ia (*aphE*, *aphD2*), Ib, Ic
APH(7")	I	Ia

* Words in parentheses indicate an alternative name of the gene.

This family includes a large number of enzymes, although they are more clinically relevant in gram-positive bacteria (Vakulenko and Mobashery, 2003). The classes and subclasses within the APHs are listed in Table 4, but only the APH(3´) enzymes are described in detail as they belong to the most extensive and best-studied class of APHs.

2.2.2.1- APH(3´)

This family is especially ubiquitous and it has been widely used asa resistance marker in molecular biology research. The APH(3´)-I subclass is found within wide host range plasmids and transposons in gram-negatives (Tenover *et al.*, 1989), and there are three enzymes belonging to this subclass that confer resistance to neomycin, paromomycin, lividomycin, ribostamycin, and kanamycin. The *aph(3´)-Ia* and *aph(3´)-Ic* genes are commonly used as marker genes in cloning vectors. The *aph(3´)-Ib* gene is part of the broad host range conjugative RP4 plasmid. The APH(3´)-II subclass includes the enzymes IIa, whose gene is also used in cloning vectors for prokaryotes and eukaryotes, IIb, encoded by a gene identified in the *P. aeruginosa* chromosome, and IIc, found in *S. maltophilia*.

The best-studied member of the APHs is the APH(3´)-IIIa, generally found in gram-positive pathogens. It confers resistance to a broad range of aminoglycosides including neomycin, lividomycin, paromomycin, butirosin, livostamycin, isepamicin, kanaycin, and amikacin. It does not modify tobramycin or gentamicin as both of them lack a free 3´-hydroxyl group. Nevertheless, it can phosphorylate other aminoglycosides that lack a 3´-hydroxyl group, such as lividomycin at position 5”. In addition, APH(3´)-IIIa is able to di-phosphorylate butirosin and neomycin B at both the 3´- and 5”- positions (McKay *et al.*, 1994). This enzyme has been crystallized and the 3D structure has been determined, revealing a close similarity to kinases from eukaryotes, therefore, the APHs are known also as “aminoglycoside kinases” (Hon *et al.*, 1997). They share almost a 40% structural identity although the share less than 5% amino acid sequence homology. Like the eukaryotic kinases, the 3D structure consists of two domains:

an N-terminal β-sheet region responsible for ATP binding, and an α-helix at the C-terminal, which acts as the aminoglycoside recognition site. The phosphate transfer occurs at the interface of the two domains, and it was suggested that the enzyme active site has evolved to "mimic" the rRNA binding site to facilitate the drug binding into this pocket before it can find its true target. Kinetic analyses have shown that this enzyme, like other kinases, requires divalent cations for its activity. Furthermore, it has been demonstrated that APH(3´)-IIIa is capable of phosphorylating several basic peptides, including protamine and myelin basic protein, although this activity is weak. Moreover, APHs can be inhibited by certain molecules that are known to inhibit kinases such as wortmannin, thus, it has been suggested that APHs evolved from protein or inositide kinases, maybe in an aminoglycoside producer where those proteins are well known.

Regarding the rest of APH(3´) enzymes, the *aph(3´)-IVa* gene is present in *Bacillus circulans* chromosome, whereas the *aph(3´)-Va*, *aph(3´)-Vb*, and *aph(3´)-Vc* genes are located in the actinomycetes chromosome. The resistance profile for the subclass V includes neomycin, ribostamycin and paromomycin, and the enzymes belonging to subclass VI confer resistance to neomycin, paromomycin, ribostamycin, butirosin, kanamycin, amikacin, and isepamicin. The only member of subclass VII, APH(3´)-VIIa, confers resistance to kanamycin and neomycin, and it was identified in *Campylobacter jejuni*.

2.2.3- Aminoglycoside O-nucleotidyltransferases (ANTs)

The ANTs represent the smallest class of aminoglycoside inactivating enzymes. These enzymes catalyze the reaction between Mg-ATP and aminoglycosides to form the O-adenylylated antibiotic molecule. To date, there are five classes of ANTs categorized depending on the position of adenylylation on the aminoglycoside molecule (Ramirez and Tolmasky, 2010): ANT(6), ANT(9), ANT(3"), ANT(2"), and ANT(4). Only one subclass exists within all of them except for ANT(4´), which includes two subclasses. The ANT(2") and ANT(3") enzymes are more frequent among gram-negative bacteria whereas the ANT(4´),

ANT(6), and ANT(9) are most usually found in gram-positive pathogens (Shaw *et al.*, 1993). The genes coding for all of these enzymes are often identified on mobile genetic elements. The most commonly found ANTs are the ANT(3") enzymes. However, they are not considered the most clinically relevant ANTs as they are characterized by specifically conferring resistance to only streptomycin and spectinomycin (Hollingshead and Vapnek, 1985).

One of the most clinically significant members of this family of enzymes is the ANT(2")-Ia, as it is a highly prevalent cause of gentamicin resistance in North America, being widespread among gram-negative pathogens. Its gene (also commonly called *aadB*) has been found amongst diverse genetic backgrounds, such as small non-conjugative plasmids, conjugative plasmids, class 1 and 2 integrons, or transposons (Ramirez *et al.*, 2005). It confers resistance to gentamicin, kanamycin, tobramycin, sisomicin, and dibekacin.

The ANT(4′)-Ia, typically found in plasmids of Staphylococci and Enterococci, is the only ANT for which the 3-D structure has been reported (Pedersen *et al.*, 1995). It revealed that this enzyme functions as a dimer, with the active site at the interface of both subunits (Figure 7). One subunit contributes to the binding of ATP and Mg^{2+}, and the other subunit is required for the transfer of the adenyl moiety to the aminoglycoside targetted. The ANT(4′)-Ia, also named *aadD*, *aadD2*, and *ant(4′,4")-I*, confers resistance to amikacin, tobramycin, and isepamicin, but also to dibekacin, which lacks a 4′- target and it is adenylated at position 4" by this enzyme.

The correlation between the modification site of certain enzymes and the aminoglycoside resistance profiles is shown in Figure 8.

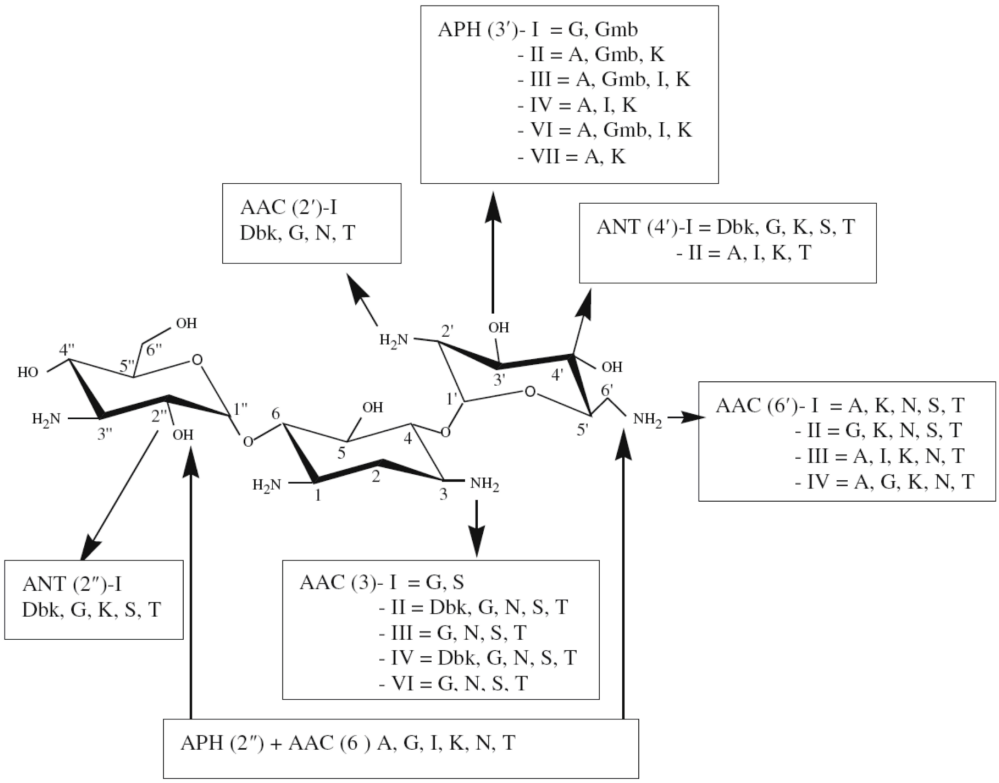

Figure 8. Modification site on the aminoglycoside molecule of selected enzymes and the resulting resistance profiles. A, amikacin; Dbk, dibekacin; G, gentamicin; Gmb, gentamicin B; I, isepamicin; K, kanamycin; N, netilmicin; S, sisomicin; T, tobramycin. Jana and Deb, 2006.

2.3- Target modification

2.3.1- Ribosomal mutations

Aminoglycoside resistance due to ribosomal mutations can occur either due to mutations of the ribosomal target (16S rRNA), or due to the mutation of ribosomal proteins (Magnet and Blanchard, 2005). Resistance to streptomycin by mutation of the ribosomal target in *M. tuberculosis* is the only case where this resistance mechanism is clinically relevant. *Mycobacterium* is the only genus of eubacteria with species that contain a single copy of the ribosomal operon, thus, a

single mutation leads to the production of a population of mutant ribosomes resulting in resistance. *rrs* is the gene that encodes the 16S rRNA, and mutations in this gene affect two highly conserved regions, the 530 loop, and the region around nucleotide 912 (*E. coli* numbering), giving rise to streptomycin resistance in *M. tuberculosis* (Dobner *et al.*, 1997). Apart from streptomycin resistance in *M. tuberculosis*, 16S rRNA mutations associated with aminoglycoside resistance have been described in clinical isolates of bacteria containing a single or low copy number of *rrs* genes. For instance, mutations at positions 1400 and 1401 have been reported in kanamycin-resistant *M. tuberculosis*, the mutation A1408G has been described in the rRNA operon of only *M. abscessus* and *M. chelonae* to be responsible for resistance to 2-DOS-containing aminoglycosides, and those mutations affecting the base pair G1064-C1192 of helix 34 have been shown to confer spectinomycin resistance in *Neisseria*.

There have been many studies using the introduction of a single *rrs* gene on a multicopy plasmid to test the effect of 16S rRNA mutants on the activity of aminoglycosides. These studies have shown that at least half of the ribosomes must be in the mutant form to confer aminoglycoside resistance (Meier *et al.*, 1994). Other works have shown how mutations leading to an allosteric change in the drug-binding pocket can be more counterproductive for aminoglycoside activity than those mutations avoiding direct contacts between the drug and the 16S rRNA.

Mutations in genes encoding ribosomal proteins can also modify the activity of aminoglycosides. Mutations in protein S12 are a cause of streptomycin resistance in *M. tuberculosis* and other species (Meier *et al.*, 1994). Although structural data showed that S12 makes direct contact with streptomycin, it has been proven that the effect of such mutations is related to conformational changes in the rRNA that prevent drug binding. Apart from mutations in the 30S ribosomal subunit, certain mutations in components of the 50S have also been associated with resistance to various aminoglycosides. For instance, mutations in the N-terminal half of S5, which contact helix 34, can confer resistance to spectinomycin as they destabilize the network of interactions in the 30S subunit.

2.3.2- Methylation of the 16S rRNA

Nowadays this strategy constitutes the most worrisome resistance mechanism to the aminoglycoside antibiotics. For this reason, as well as for composing the main topic of this Thesis, it will be extensively described in the next Chapter.

CHAPTER 3. The 16S rRNA methyltransferases: an aminoglycoside high-level resistance mechanism.

Despite the continuous usefulness of aminoglycoside antibiotics since their discovery in 1944, the never-ending decrease of the development of new molecules combined with the increasing emergence of resistant strains has turned the resistance against this family of antibiotics into the central topic of numerous research works.

Until now, we have mentioned several types of aminoglycoside resistance mechanisms, of which the inactivation of the antibiotic molecule mediated by enzymes is the most common. Even so, those mechanisms do not confer high-level resistance as they usually depend on various factors such as the amount of drug-inactivating enzyme produced. Moreover, they generally only have a few specific substrates so a coexistence of several complementary determinants is needed for a broader spectrum of activity. Regarding this point, when resistance is due to target modification, high levels of resistance to aminoglycosides can be achieved. As described before, modification of the target due to a ribosomal mutation rarely occurs and is only clinically significant in *M. tuberculosis*. However, there is a target-site modification in which the bacterium protects the target by employing enzymes that add a methyl group to specific nucleotides in the 16S rRNA that are essential for aminoglycoside binding, thus, inhibiting the antibiotic action without interfering with other ribosomal functions. This mechanism has become a major concern regarding aminoglycoside resistance, not only because high levels of resistance to most clinically relevant aminoglycosides can be achieved, but due to the potential of both the vertical and horizontal transfer of the genes encoding these enzymes.

3.1- Housekeeping 16S rRNA methyltransferases in aminoglycoside producers

Most antibiotics used in clinical settings are microbial secondary matabolites, and although there are various antibiotic-producing prokaryotes, the most prolific are the actinomycetes. Hence, antibiotic-producing organisms must be able to protect themselves against the toxic effects of their own products. This self-defence can be accomplished in different ways, from target-based protection to efflux systems or any other strategy that prevents the drug-target interaction (Cundliffe and Demain, 2010).

Self-defence involving the antibiotic target sites is widespread and it commonly involves ribosomes as most drugs that inhibit protein synthesis bind to specific sites located on any of the ribosomal subunits. These sites are principally comprised of rRNA, thus, self-resistance via site-specific methylation of rRNA is a widespread tactic used by those microorganisms producing agents that act on the ribosome (Savic *et al.*, 2009). This does not only include aminoglycosides, but also other compounds such as macrolides, lincosaminides, or thiopeptides. However, ribosome-based resistance has not been found in all organisms that produce ribosome inhibitors, as is the case with producers of streptomycin, neomycin, tetracycline, or chloramphenicol (Cundliffe and Demain, 2010). The high-level resistance achieved by this ribosomal modification is generally produced by the action of a single rRNA methyltransferase that acts at a specific site characteristic of a given phenotype, even when it is a complex pattern. One example is the "MLS" phenotype (causing resistance to macrolides, lincomycin, and streptogramins), which is widespread among actinomycetes. Nevertheless, there are exceptions where rRNA methyltransferases act together, conferring resistance for example to tylosin in *Streptomyces fradiae*, or to avilamycin in *Streptomyces viridochromogenes* (Cundliffe and Demain, 2010).

Ribosomal modifications resulting in aminoglycoside self-resistance involve monomethylation of the 16S rRNA at one of two sites: G1405 or A1408 (*E. coli* numbering). A substitution occurs on the nitrogen ring to generate 7-methylguanosine or 1-methyladenosine, respectively, blocking the binding of certain aminoglycosides to the ribosome (Savic *et al.*, 2009).

Several MTs conferring aminoglycoside resistance in their producers have been described so far, mainly from different species of the genus *Streptomyces*, *Micromonospora,* and to a lesser extent, *Saccharopolyspora* (Table 6) (Demydchuk *et al.*, 1998; Kojic *et al.*, 1992; Ohta *et al.*, 1993). These MTs are included in the *S*-adenosyl-L-methionine (SAM)-dependent RNA enzyme superfamily and they have been classified in two groups of enzymes named the Kgm and Kam families (Conn *et al.*, 2009). This classification is based on their target residue by ascertaining the site of methylation on the 16S rRNA by Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). The Kgm (kanamycin-gentamicin MTs) family methylate residue G1405 whereas the Kam (kanamycin-apramycin MTs) family methylate the A1408 residue (Husain *et al.*, 2010; Macmaster *et al.*, 2010; Savic *et al.*, 2009). Comparison of antibiotic resistance patterns between both families unambiguously identifies functional differences correlating with the modifications at G1405 and A1408. All Kgm family members confer high-level resistance to 4,6-DOS aminoglycosides (such as kanamycin and gentamicin), with MIC values over 1000 mg/L. However, they do not reduce susceptibility to 4,5-DOS (neomycin, paromomycin) nor to other aminoglycosides such as apramycin and streptomycin. On the other hand, the Kam MTs do not confer resistance to gentamicin but they increase resistance levels to neomycin as well as they confer high-level resistance to kanamycin and apramycin.

Table 6. Self-aminoglycoside resistance via methylation of 16S rRNA

Aminoglycoside	Producer	Gene	MT family
Gentamicin	*M. purpurea*	*gmrA*	Kgm (methylate G1405)
Kanamycin	*S. kanamyceticus*	*Kmr*	
Nebramycin complex	*S. tenebrarius*	*kgmB*	
Fortimicin A	*M. olivasterospora*	*fmrO*	
Istamycin	*S. tenjimariensis*	*kamA*	Kam (methylate A1408)
Nebramycin complex	*S. tenebrarius*	*kamB*	
Sporaricin	*Sac. hirsuta*	*kamC*	

Genes of the Kgm and Kam families are typically present in respective antibiotic-biosynthesis clusters. Hence, *grmA*, *kmr*, and *kamA* are found within the gentamicin, kanamycin and istamycin biosynthesis clusters of *M. purpurea*, *S. kanamyceticus*, and *S. tenjimariensis*, respectively (Table 6). However, *S. tenebrarius* bears *kgmB* and *kamB* genes, belonging to the MT families Kgm and Kam, respectively. This is explained because *S. tenebrarius* is a producer of the so called "nebramycin complex", which includes different aminoglycosides (apramycin and kanamycin B derivatives including tobramycin). The phylogenetic relationship between some of the MTs responsible for self-resistance in aminoglycoside producers is shown in Figure 9.

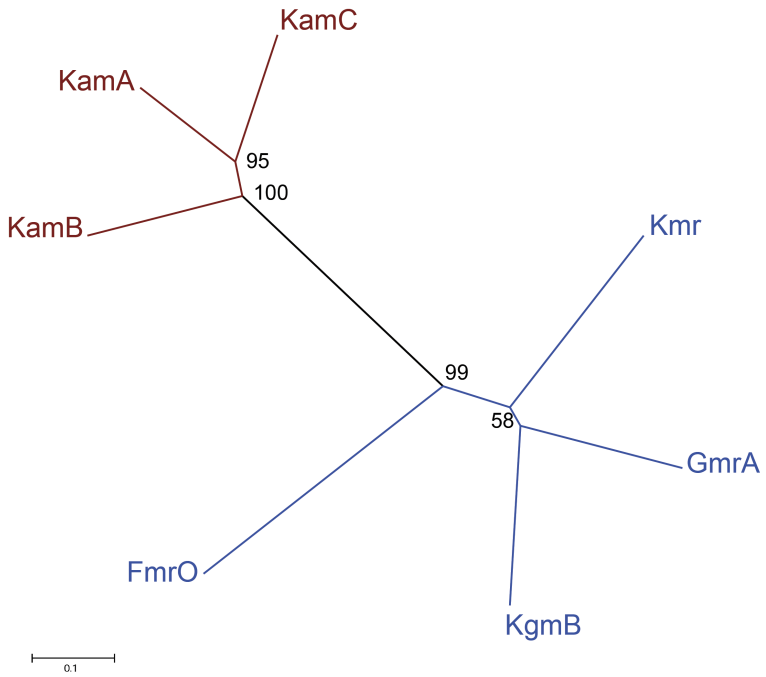

Figure 9. A dendrogram with relevant intrinsic 16S rRNA MTs from AG producers. A clear differentiation can be observed between the Kgm (blue) and the Kam (red) family members. The bar denotes genetic distance. Bootstrap values are the result of 1000 iterations.

3.2- Acquired 16S rRNA methyltransferases in gram-negative bacteria

It is known that large amounts of aminoglycosides have been used in the last decades for the treatment of infections caused by mainly gram-negative but also gram-positive bacteria. On the other hand, during this period of time, these bacteria have acquired several resistance mechanisms to inhibit aminoglycosides action. Among these mechanisms, as described above, the most frequently found was the enzymatic modification of the aminoglycoside molecule. Despite ribosomal protection via methylation being widely described as a self-defence strategy in aminoglycoside-producers since the 80's, until recently, this resistance mechanism was believed to be absent in clinically relevant bacteria. Nowadays, the methylation of the ribosomal target responsible for high-level aminoglycoside resistance is an emerging mechanism of great concern in clinically relevant gram-negative bacteria.

3.2.1- General traits of acquired 16S rRNA MTs

3.2.1.1- First description and nomenclature

In 2003, an acquired 16S rRNA MT was initially reported (Galimand *et al.*, 2003). A *Klebsiella pneumoniae* isolated in 2000 from a urinary tract infection in France was found to be highly resistant to all 4,6-DOS aminoglycosides and fortimicin. The genetic determinant responsible for that phenotype was cloned into a laboratory *E. coli* strain. The inserted sequence was compared with sequences in the GenBank data library, revealing sequence identity with a fragment of a plasmid sequence from *Citrobacter freundii* in Poland (pCTX-M3), which had been deposited in GenBank in 2002 under the accession number AF550415. There was an ORF within that fragment of which the deduced aminoacid sequence shared similarity (27%) with a 16S rRNA methyltransferase from *Streptoalloteichus hindustanus*. However, even though the whole nucleotide sequence of pCTX-M3 had been previously elucidated, this probable 16S rRNA MT escaped

uncharacterized and unpublished until Galimand and colleagues named this new protein ArmA for aminoglycoside resistance methyltransferase A. It showed homology with those aminoglycoside resistance MTs from actinomycetes (identity levels of ArmA with these proteins ranging from 21% to 30%).

Only 4 months after the report of ArmA, another article describing a 16S rRNA MT in a gram-negative bacterium was published (Yokoyama *et al.*, 2003). The 756 bp gene, designated *rmtA* (ribosomal RNA methyltransferase A), was identified in a *P. aeruginosa* strain isolated from a clinical sample in 1997 in Japan. This gene was found to confer high-level resistance to arbekacin, amikacin, gentamicin, kanamycin and tobramycin when cloned and expressed in *E. coli* and *P. aeruginosa*.

RmtB and RmtC acquired MTs were subsequently discovered for the first time also from Japanese hospitals. The first one was isolated from a *Serratia marcescens* strain in 2002, and published in 2004 (Doi *et al.*, 2004). The resulting amino acid sequence of *rmtB* shared 82% identity with RmtA, and a methylation assay with histidine-tagged recombinant protein confirmed that RmtB was a functional 16S rRNA MT. The first identification of RmtC was published in 2006 (Wachino *et al.*, 2006b). It was found in a *Proteus mirabilis* strain isolated in 2003, and it was also confirmed to be a 16S rRNA MT conferring the same resistance pattern as ArmA, RmtA, or RmtB.

Successively, other 16S rRNA MTs have been discovered among gram-negative bacteria to date. RmtD was firstly reported in 2007 from a clinical *P. aeruginosa* strain in Brazil (Doi *et al.*, 2007c), and its variant RmtD2, which shows 9 amino acid substitutions compared to RmtD, was found in Argentina in 2011 (Tijet *et al.*, 2011). The first and sole identification of the RmtE methyltransferase was published in 2010. It was found in *E. coli* bovine isolates from the United States (Davis *et al.*, 2010). RmtF was identified in a *K. pneumoniae* clinical strain in Reunion Island in 2012 (Galimand *et al.*, 2012), and RmtG and RmtH have been published in 2013 in *K. pneumoniae* isolates from Brazil and Iraq, respectively (Bueno *et al.*, 2013; O'Hara *et al.*, 2013). All the above mentioned methyltransferases are considered to be (or have been confirmed to be) N7-G1405

16S rRNA MTs. With the exception of NpmA, which was reported in 2007 in a clinical *E. coli* strain from Japan, and it was confirmed to methylate the 16S rRNA at position A1408. Additionally, it shows an appropriate resistance phenotype according to its function (Wachino *et al.*, 2007). Nevertheless, this was the only time NpmA has been identified, and no other N1-A1408 MT has been reported in gram-negative bacteria to date.

Regarding the nomenclature of these acquired 16S rRNA MTs in gram-negative bacteria, due to their emergence and increasing prevalence, in 2008, Doi and colleagues proposed some rules in order to prevent confusion over the nomenclature of 16S rRNA methylases (Doi *et al.*, 2008b), to avoid cases such as the two existing nomenclature systems for aminoglycoside acetyltransferases. Hence, the following guidelines were suggested: a gene with an amino acid identity greater than 95% with the closest known 16S rRNA MT will be assigned a variant number starting from two (e.g. *rmtD2*). A gene that has between 50 and 95% amino acid identity with the closest known 16S rRNA MT will be assigned a new alphabet letter according to the closest existing gene name (e.g. *rmtF*, *armB*). A gene that either shows an amino acid identity of less than 50% with the closest known 16S rRNA MT or that is proven to methylate a new residue of 16S rRNA, may be assigned a completely new gene name (i.e, *npmA*).

3.2.1.2- Clinical relevance and resistance phenotypes conferred by the 16S rRNA MTs

Despite the emergence of antimicrobial resistance, AGs retain broad-spectrum activity against multidrug-resistant gram-negative bacilli. Thus, AGs often remain a useful weapon against those pathogens resistant to the first choice antibiotics. Even though modifying enzymes represent the most prevalent mechanism of AG resistance, many strategies have been employed to overcome the function of these enzymes. For instance, amikacin has been generally reserved for AG resistant pathogens because it escapes inhibition by most AMEs. Furthermore, arbekacin, available in Japan for the treatment of MRSA infections, is usually

stable despite enzymatic modification with the sole exception of the bifunctional enzyme AAC(6′)-APH(2′′). This modification, however, confers only low-level resistance to arbekacin (MIC between 4 and 32 mg/L).

Regardless of AMEs being the most frequent AG resistance mechanism, acquired 16S rRNA MTs rapidly came into the spotlight since their first identifications due to several factors (Doi and Arakawa, 2007). These include their expression by genes that are carried by mobile genetic elements or their association with other important resistance mechanisms, such as the recently described NDM-1 carbapenemase). The main alarming factor was related to their function, since these acquired 16S rRNA MTs confer resistance to all AGs available in clinical settings, aditionally the 16S rRNA MTs-producing bacteria are able to grow under very high concentrations of AGs as compared with the MIC values detected for various AMEs. Even though it cannot be stated that acquired 16S rRNA MTs are only found in pathogenic bacteria, as they can also be found in gram-negative organisms that belong to the comensal microbiota, they are commonly detected in gram-negative pathogens isolated from nosocomial and community acquired infections (Wachino and Arakawa, 2012). Moreover, some of the most prevalent bacterial species reported to produce 16S rRNA MTs (as for instance *Klebsiella* spp., *A. baumanii*, *P. aeruginosa*, and *Enterobacter* spp.) are encompassed in the so called ESKAPE pathogens. This group comprises multi-drug resistant strains of *E. faecium*, *S. aureus*, *Klebsiella* spp., *A. baumannii*, *P. aeruginosa*, and *Enterobacter* spp., and according to data from the Centers for Disease Control, the six ESKAPE bacteria are responsible for two-thirds of all healthcare associated infections (Rice, 2008).

The N7-G1405 16S rRNA MTs (ArmA, RmtA-H) are able to confer high-level resistance to 4,6-disubstituted DOS, such as gentamicin, amikacin, tobramycin, kanamycin, or even arbekacin. However, they do not reduce the susceptibility to apramycin (4-monosubstituted DOS), 4,5-disubstituted DOS, or streptomycin (non DOS) (Table 7). As a rule, when these genes are cloned and expressed in *E. coli* laboratory strains, the MIC values are ≥ 128 mg/L for all the 4,6-disubstituted DOS, even though some differences among all the existing

publications to date can be found. It is not infrequent to find MICs >1024 mg/L to different 4,6-disubstituted DOS, much higher than the MIC breakpoints stated by EUCAST (where an MIC > 16 mg/L is considered resistant for amikacin, and an MIC > 4 mg/L is resistant to gentamicin and tobramycin). Nonetheless, in most of the reports the MIC of tobramycin is slightly lower as compared to other 4,6-disubstituted DOS. In addition, wild-type strains bearing a N7-G1405 16S rRNA MT generally show MIC values > 1024 mg/L for all 4,6-DOS due to the co-existence of the methyltransferase with some AMEs within the same strain.

In contrast, the only N1-A1408 16S rRNA MT identified to date in gram-negative bacteria, NpmA, confers high-level resistance to the 4,5-disubstituted DOS (neomycin), 4-monosubstituted DOS (apramycin), but it also significantly reduces susceptibility to the 4,6-disubstituted DOS (Wachino *et al.*, 2007) (Table 7). This means that this type of MT is of even greater concern as it confers a broader range of aminoglycoside resistance.

Table 7. Resistance phenotype conferred by acquired 16S rRNA MTs

Aminoglycosides	**Acquired 16S rRNA MTs**	
	N7-G1405	**N1-A1408**
4,6-DOS	RR	R
4,5-DOS	S	RR
Apramycin	S	RR
Streptomycin	S	S

RR, high-level resistance (MIC >128 mg/L); R, resistant (MIC >16 mg/L and < 128 mg/L); S, susceptible.

Regarding the screening procedure of 16S rRNA MT-producing bacteria, since the first identifications of these acquired enzymes, those strains showing resistance to AGs started to be further characterized by assaying their ability to grow in the presence of high concentrations of various AGs, as testing with only 1 or 2 antibiotics would not be conclusive due to the AMEs. If we check among all

the existing reports on acquired MTs, an isolated strain is commonly considered to be a potential carrier of an N7-G1405 16S rRNA MT when it is able to grow with a concentration of different 4,6-DOS greater than 128 mg/L, but it remains susceptible to the 4,5-DOS and apramycin. Some authors proposed arbekacin as a useful AG to test for the presence of these MTs (Doi *et al.*, 2004; Doi *et al.*, 2007b), as it is only modified by the bifunctional enzyme AAC(6´)-APH(2´´) (Fujimura *et al.*, 2000). However, the combination of gentamicin and amikacin is the most frequently used for testing purposes. Along the same line, screening for an N1-A1408 16S rRNA MT would require a consistent phenotype of resistance to 4,6-DOS and high-level resistance to 4,5-DOS and apramycin (Table 7). Furthermore, the identification of these MTs is more complex as its resistance profile against the clinically utilized AGs is very similar to that confeered by the co-production of multiple AMEs. Moreover, the high-level resistance to apramycin can be confused with the presence of AAC(3)-IV, which is the most prevalent gene for apramycin resistance in *E. coli.* Anyhow, phenotypic screening provides an initial approach and the only confirmatory method at present to detect the known 16S rRNA MTs is PCR. All the aforementioned procedures are generally performed with isolates of *Enterobacteriaceae*, *P. aeruginosa*, and *A. baumanii*, and it is important to take the possibility of intrinsic high-level resistance not due to methylation in certain opportunistic bacteria into account, such as *Stenotrophomonas maltophilia.*

3.2.1.3- Intrinsic vs. acquired 16S rRNA MTs

Based on sequence similarity, all MTs intended to methylate residue G1405 on the 16S rRNA are categorized as 16S rRNA MTs with an FmrO domain. However, the classification of 16S rRNA MTs into four distinct families depending on their origin and target has been previously proposed (Conn *et al.*, 2009): “Kgm” family (kanamycin-gentamicin MTs) are intrinsic MTs of residue G1405, “Arm” family (aminoglycoside resistance MTs) are acquired MTs of G1405, “Kam” family (kanamycin-apramycin MTs) are intrinsic MTs targetting A1408, and “Pam” family (pan-aminoglycoside resistance MTs) are acquired MTs of residue

A1408. Thus, those acquired methyltransferases belonging to the Arm family (ArmA, RmtA-H) would be the equivalent to the Kgm family of the producers, and NpmA (Pam family) would be in the Kam family MTs.

Concerning the phylogenetic relatedness of the acquired 16S rRNA MTs, the percentage of amino acid identity between the Arm family members (ArmA, RmtA-H) range from 20% to 81%, whereas NpmA shares a low identity with MTs of the Arm family (Table 8).

Table 8. Percentage of identity among amino acid sequences of acquired 16S rRNA MTs

	Amino acid sequence identity (%)									
16S rRNA MT	ArmA	RmtA	RmtB	RmtC	RmtD/D2	RmtE	RmtF	RmtG	RmtH	NpmA
ArmA	100	27	27	**29**	20/25	27	22	24	26	10
RmtA		100	**81**	**30**	39	**35**	42	35	63	**12**
RmtB			100	28	39/38	32	42	35	**64**	11
RmtC				100	27/29	28	26	22	28	5
RmtD/D2					100/96	31/32	**44/46**	**57/59**	38	9
RmtE						100	32	28	28	5
RmtF							100	36	39	2
RmtG								100	30	5
RmtH									100	5
NpmA										100

Relatedness among all the acquired MTs. The highest similarities for each MT are highlighted.

When comparing the amino acid identity shared by all the 16S rRNA MTs, the G1405 methyltransferases cluster together, and so do those methylating the position A1408 (Figure 10), however, the acquired MTs share less than 33% amino acid identity with the equivalent group of intrinsic MTs. In relation to the G+C contents of 16S rRNA MTs genes, most MT genes from aminoglycoside-producing isolates (gram-positive) have a G+C content ranging from 64% to 72% (Liou *et al.*, 2006), whereas the G+C content of the first reported acquired MT genes is around

30%-59%. Therefore it was hypothesized that the origin of the acquired MTs is unlikely to be the aminoglycoside-producing actinomycetes (Liou *et al.*, 2006). However, there are certain low G+C content gram-positive bacilli able to produce aminoglycosides (i.e., *B. circulans*). As it is known that genes from gram-negative bacteria are not generally expressed in gram-positive organisms, Liou and colleagues cloned and expressed *armA* with its putative promoter in *Bacillus subtilis* where it conferred resistance, giving rise to the conjecture of how *armA* could have originated from aminoglycoside-producing low G+C content gram-positive bacteria (Liou *et al.*, 2006). Nonetheless, the origin of these acquired 16S rRNA MTs remains unknown.

Regarding the determination of the target nucleosides for both groups of 16S rRNA MTs (intrinsic and acquired), there have been already several studies with the aim of ascertaining the exact position these MTs methylate (Beauclerk and Cundliffe, 1987; Gutierrez *et al.*, 2012; Holmes and Cundliffe, 1991; Liou *et al.*, 2006, Savic *et al.*, 2009). In general, this is experimentally performed by primer extension methodology, which makes it possible to determine the methylated residue (N7-G1405, N1-A1408), and Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), which can measure the presence or absence of a methyl group (by reading a mass difference of 14 Da.) and therefore confirm if a methylation occurs (Gutierrez *et al.*, 2012). The methylation sites have been assessed for some intrinsic 16S rRNA MTs, such as KgmB (G1405), Sgm (G1405), GrmA (G1405), Krm (G1405), KamA (A1408), and KamC (A1408). In relation to the acquired 16S rRNA MTs, N7-G1405 has been confirmed to be the methylated residue for ArmA, RmtB, RmtC, and RmtF, whereas NpmA has been determined to methylate at position N1-A1408. These acquired enzymes have a methyltransferase activity specific for the bacterial 30S ribosomal subunit consisting of 16S rRNA and several ribosomal proteins, but not for the naked 16S rRNA, which indicate that ribosomal proteins play a crucial role in the precise substrate recognition. The methylation step takes place before the binding of the two ribosomal subunits (Wachino *et al.*, 2010).

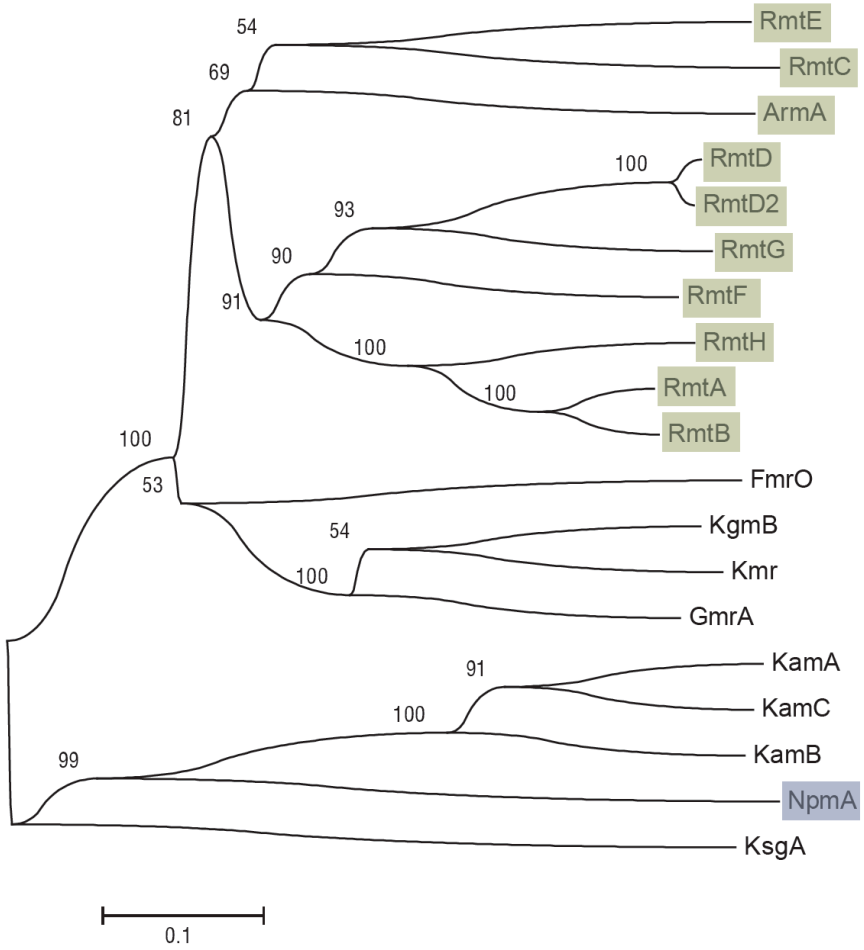

Figure 10. Dendrogram with the acquired 16S rRNA MTs and a selection of AG resistance MTs from the producers, as well as the indigenous MT of *E. coli*, KsgA. Green: acquired MTs of the G1405; blue: acquired MT of the A1408. The bar denotes genetic distance. Bootstrap values are the result of 1000 iterations.

3.2.2- Epidemiology of acquired 16S rRNA MTs

3.2.2.1- Prevalence and distribution

Despite data on the prevalence of aminoglycoside resistance mediated by 16S rRNA methylation in gram-negative bacteria remaining scarce for the first 3 or 4 years following their first identification, an increase in available data has now

been observed, mainly due to the existence of surveillance programs. Information obtained from global antimicrobial surveillance studies is important to establish trends in antimicrobial resistance in bacteria, although local studies constitute a significant part of the recovered data. Surveillance programs vary widely in that some programs focus on specific pathogens, whereas other programs focus on specific antimicrobial agents. One of the most important surveillance programs of antimicrobial resistance is the SENTRY Antimicrobial Surveillance Program. This is an ongoing international program that was initiated in 1997 to monitor the occurrence and antimicrobial susceptibility of bacterial pathogens causing nosocomial and community-acquired infections (Masterton, 2008). Sentinel sites are present in more than 30 countries worldwide, and this program has generated more than 180 publications thus far.

Although resistance mediated by 16S rRNA MTs has been increasingly reported worldwide (Figure 11), the distribution of these MTs is still poorly defined, with little molecular epidemiology data from recent collections. Nevertheless, acquired 16S rRNA MTs seem to be most prevalent in Asian countries. SENTRY data for 2007-2008 indicated prevalence rates of aminoglycoside resistance due to methylation among *Enterobacteriaceae* of 10.5% in India, 6.9% in China, 6.1% in Korea, 5% in Taiwan, and 3.1% in Hong Kong (Livermore *et al.*, 2011). However, there are many reports generated at the local level from different hospitals and institutions worldwide, and regardless of the variability observed between hospitals, when taken as a whole, the highest prevalence is found in Asian countries. Additionally, local surveys have provided useful data describing the role of reservoirs such as animals or the environment in the distribution of acquired 16S rRNA MTs, as well as on the molecular genetics involved in their spread. Surprisingly, there are not as many reports from America as there are in Asia or Europe, and they are generally associated with non-fermentative gram-negative bacilli (*A. baumannii*, *P. aeruginosa*) (Doi *et al.*, 2007b; Fontes *et al.*, 2011). Little information is available on the distribution of acquired MTs in Africa, as up to now there are only 3 publications from Algeria and Kenya (Bouzidi *et al.*, 2011; Naas *et al.*, 2011; Poirel *et al.*, 2011c).

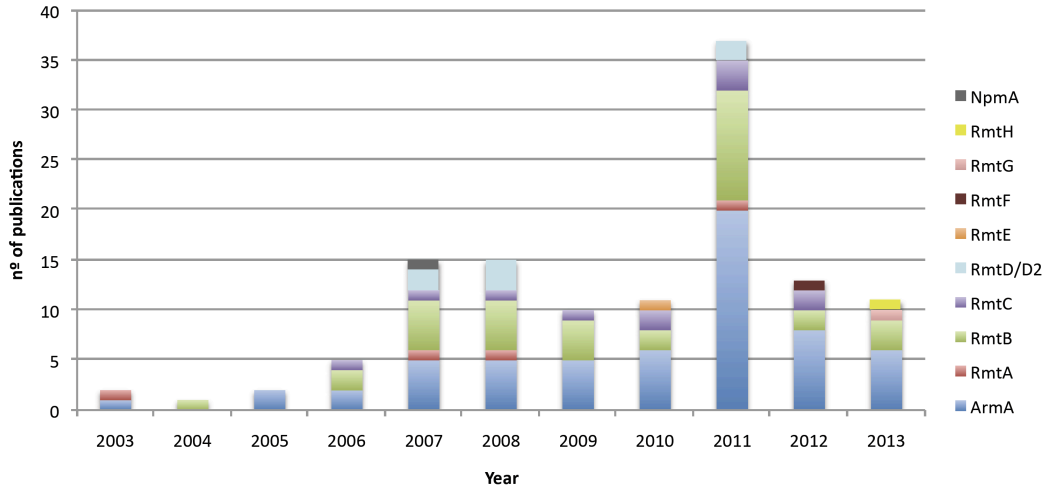

Figure 11. A graph representing the number of publications concerning each acquired 16S rRNA MT per year since their first identification in 2003.

When comparing the global prevalence of the known acquired 16S rRNA MTs, an evident division can be observed. Hence, ArmA and RmtB have been the most prevalent MTs by far, whereas RmtA, the one firstly identified with ArmA, has only been reported 4 times (Yokoyama *et al.*, 2003; Yamane *et al.*, 2007; Jin *et al.*, 2009; Poirel *et al.*, 2011b), and RmtE, RmtF, RmtG, RmtH, and NpmA, have been only identified once (Wachino *et al.*, 2007; Davis *et al.*, 2010, Galimand *et al.*, 2012; Bueno *et al.*, 2013; O'Hara *et al.*, 2013). RmtC and RmtD are considered MTs with a low prevalence, however, there has been a clear increase in the number of publications reporting RmtC in the last years (Figure 11). Commonly, a single acquired MT is produced by one strain, although several reports have described the co-production of both ArmA and RmtB, or ArmA and RmtC by the same isolate (Ma *et al.*, 2009, Mushtaq *et al.*, 2011).

Concerning the highly prevalent acquired MTs, ArmA has been reported in several Asian and European countries, Algeria in Africa, Australia, and in the USA (Galimand *et al.*, 2005; Gonzalez-Zorn *et al.*, 2005b; Bogaerts *et al.*, 2007; Folster *et al.*, 2009; Ma *et al.*, 2009; Poirel *et al.*, 2010; Karah *et al.*, 2011; Livermore *et*

al., 2011a; Moissenet *et al.*, 2011; Naas *et al.*, 2011; Poirel *et al.*, 2011d; Samuelsen *et al.*, 2011; Sekizuka *et al.*, 2011; Sung *et al.*, 2011; Zacharczuk *et al.*, 2011; McGann *et al.*, 2012; Guo *et al.*, 2012; Mezzatesta *et al.*, 2013; Saule *et al.*, 2013; Sonnevend *et al.*, 2013; Tada *et al.*, 2013c) (Figure 12). In Asia, it has been found in Japan, South Korea, China, Taiwan, Vietnam, Pakistan, Bangladesh, India, Afghanistan, Oman, and in the United Arab Emirates. Amongst these, the prevalence of ArmA is highest in South Korea, Taiwan, China, and Vietnam. In Europe, ArmA is present in Spain, France, Italy, Belgium, United Kingdom, Norway, Sweden, Latvia, Serbia, Poland, and Bulgaria, the last two reaching the highest prevalence rates. RmtB distribution is also scattered worldwide, although as compared with ArmA, RmtB prevalence and spread in Europe is low and it has only been found in France, Belgium, and Greece (Berçot *et al.*, 2008; Périchon *et al.*, 2008; Galani *et al.*, 2011). In addition, it has been occasionally isolated in the USA, Mexico, Brazil, Australia, Turkey, Bangladesh, Pakistan, Vietnam and Japan, while it is highly prevalent in China, South Korea, and Taiwan (Doi *et al.*, 2004; Fritsche *et al.*, 2008; Ma *et al.*, 2009; Park *et al.*, 2009; Berçot *et al.*, 2010; Poirel *et al.*, 2010; Bogaerts *et al.*, 2011; Deng *et al.*, 2011a, 2011b; Poirel *et al.*, 2011; Tada *et al.*, 2013). Among the lowly prevalent acquired MTs, RmtC has only been found a few times but its distribution is spread out (i.e. USA, United Kingdom, Kenya, India, Bangladesh, Japan, Australia and New Zealand) (Wachino *et al.*, 2006; Zong *et al.*, 2008; Folster *et al.*, 2009; Hopkins *et al.*, 2010; Poirel *et al.*, 2011c; Islam *et al.*, 2012; Williamson *et al.*, 2012), whereas the few isolates bearing RmtA are mostly gathered around Asia (India, South Korea, and Japan) (Yokoyama *et al.*, 2003; Jin *et al.*, 2009; Poirel *et al.*, 2011b), and those with RmtD/D2 have only been found in South America (Chile, Argentina, and Brazil) (Fritsche *et al.*, 2008). Moreover, RmtD is considered to be highly prevalent in Brazil (Doi et al., 2007d).

Figure 12. Map indicating the global distribution and prevalence of the acquired 16S rRNA MTs from their first identification until now. Isolates belonging to those publications that constitute this Thesis are not included. Those cases where alternative sources other than humans are represented (e.g. pigs, chickens, cattle, pets, food, or environment) means that one or more isolates (even all of them) are from that source.

3.2.2.2- Reservoirs and routes of transmission

After so much literature on how aminoglycoside-producing bacteria avoid death by methylation of the 16S rRNA, an evident question arose in the field of aminoglycoside resistance: why this mechanism had not been found in clinically relevant bacteria exposed to aminoglycosides? There was no certainty of whether it did not exist or it was simply missed because of the screening methods, which may lead it to be confused with the production of multiple AMEs. Once ArmA and RmtA were initially reported in 2003 from strains of *K. pneumoniae* and *P. aeruginosa*, respectively, and they were confirmed to confer a high-level resistance to all 4,6-DOS when cloned and expressed in *E. coli*, an in depth search for 16S rRNA MTs in different gram-negative bacilli originated.

The most prevalent and distributed enzymes, ArmA and RmtB, have generally been found among species of *Enterobacteriaceae* (Table 9). In addition, ArmA and RmtB have occasionally been isolated from non-fermentative gram-negative bacilli, such as *P. aeruginosa* or *A. baumanii*, which are nosocomial

pathogens of global concern (Gurung *et al.*, 2010; Tada *et al.*, 2013c). In the case of RmtA or RmtD, most strains found are *P. aeruginosa* as compared with the other acquired MTs, which are mostly detected in *Enterobacteriaceae* strains (Doi *et al.*, 2007b; Jin *et al.*, 2009). For a long time, RmtC was thought to be present only in *P. mirabilis*, as it was initially identified in a *P. mirabilis* strain in Japan (Wachino *et al.*, 2006b), two years later in *P. mirabilis* from Australia (Zong *et al.*, 2008), and then there were no more reports until its discovery in *Salmonella enterica* isolates (Hopkins *et al.*, 2010). Acquired 16S rRNA MTs have only been found in gram-negative bacteria, but not in gram-positives. This led to the question of whether or not these enzymes are functional in gram-positive bacteria. Hence, two laboratories have confirmed that ArmA and RmtC confer high-level resistance to 4,6-DOS AGs when cloned and expressed in *B. subtilis*, and *B. subilis* and *S. aureus*, respectively (Liou *et al.*, 2006; Wachino *et al.*, 2010).

The acquired 16S rRNA MTs have mostly been identified in human strains isolated from either nosocomial or community-acquired infections. Nonetheless, the early detection of ArmA in a single *E. coli* isolated from a farm pig (Gonzalez-Zorn *et al.*, 2005b) raised the question of whether other sources such as animals or food products can be involved in the origin or spread of acquired 16S rRNA MTs. ArmA, RmtB, and RmtE are the only MTs identified from animals to date. ArmA has been reported from pig isolates in Spain and South Korea (Gonzalez-Zorn *et al.*, 2005b; Choi *et al.*, 2011), from cattle in South Korea (Choi *et al.*, 2011), and from chickens in China and South Korea (Choi *et al.*, 2011; Du *et al.*, 2012) (Figure 12). In China, RmtB has been widely associated to pigs, chickens, and cattle, but also livestock-farming environments and pet animals (Deng *et al.*, 2011a, 2011b; Li *et al.*, 2012; Du *et al.*, 2012) (Figure 12). The only description of RmtE was from *E. coli* recovered from cattle (Davis et al., 2010). Regarding other sources, RmtD was detected in a bacterium isolated from a Brazilian river (Fontes et al., 2011), additionally RmtC was found in a *S. enterica* sample collected from food in the UK (Hopkins *et al.*, 2010) (Figure 12). Moreover, both the strains recovered from a river and from food were clonally related to the human strains carrying the respective MT, indicating a clear link between the different sources of transmission for this resistance mechanism.

Table 9. Bacterial species in which the acquired MTs had been found to date*

Acquired 16S rRNA methyltransferase	Bacterial species
ArmA	*Klebsiella pneumoniae, Klebsiella oxytoca, Escherichia coli, Enterobacter aerogenes, Enterobacter cloacae, Serratia marcescens, Citrobacter freundii, Citrobacter amalonaticus, Proteus mirabilis, Shigella flexneri, Salmonella enterica, Providencia stuartii, Pseudomonas aeruginosa, Acinetobacter baumanii, Acinetobacter calcoaceticus*
RmtA	*P. aeruginosa, K. pneumoniae*
RmtB	*E. coli, K. pneumoniae, E. cloacae, E. aerogenes, Enterobacter amnigenus, P. mirabilis, C. freundii, S. marcescens, A. baumannii*
RmtC	*P. mirabilis, S. enterica, E. coli, K. pneumoniae, P. stuartii*
RmtD/D2	*P. aeruginosa* (D), *K. pneumoniae* (D, D2), *E. cloacae* (D, D2), *Enterobacter agglomerans* (D2), *C. freundii* (D, D2), *P. mirabilis* (D2)
RmtE	*E. coli*
RmtF	*K. pneumoniae*
RmtG	*K. pneumoniae*
RmtH	*K. pneumoniae*
NpmA	*E. coli*

* Isolates from the publications constituting this Thesis are not included

3.3- Genetic background of acquired 16S rRNA methyltransferases

3.3.1- Location of the 16S rRNA MT genes

One of the issues that raises concerns about the known acquired 16S rRNA MTs is that their structural genes are generally located within transferable plasmids instead of the chromosome, which leads to the possibility of vertical but also horizontal transfer, thus accelerating their spread. The *armA* and *rmtB* genes have

not been found chromosomally located thus far, regardless of the high number of strains that harbor these methyltransferases. In addition, the few reports of *rmtA* and the single identification of *npmA* proved that these genes are borne by conjugative plasmids (Yokoyama *et al.*, 2003; Yamane *et al.*, 2007; Wachino *et al.*, 2007; Jin *et al.*, 2008). On the other hand, *rmtE*, *rmtF* and *rmtG* have been discovered on plasmids that cannot be transferred by conjugation (Davis *et al.*, 2010; Galimand *et al.*, 2012; Bueno *et al.*, 2013). The only work describing *rmtH* states that it is likely located on the chromosome. Interestingly, *rmtD*, which is highly prevalent in South America, has always been found to be chromosomally located among various clonally related *P. aeruginosa* strains, whereas *rmtD2* was observed to spread in Argentina among non-related strains and could be transferred by conjugation. *rmtC* was initially found to be borne by a plasmid in a *P. mirabilis* strain, but was pointed out to be chromosomally located in a *S. enterica* clone spread in the UK.

3.3.1.1- Plasmids carrying acquired 16S rRNA MTs

In most cases acquired 16S rRNA MTs are plasmid-mediated. Classifying plasmids into groups based on their phylogenetic relatedness is helpful to analyze their distribution but also to assess their relationship to host cells (Carattoli, 2009). In 1971, Datta and Hedges proposed a plasmid categorization scheme on the basis of plasmid stability during conjugation (Datta and Hedges, 1971). They defined the term “Incompatibility” as the inability of two related plasmids that share a common replication control to be propagated in the same cell line, and therefore those plasmids would belong to the same “Incompatibility group”. Since 2005, a PCR-based replicon typing scheme has been available to target the replicons of the major plasmid families occurring in *Enterobacteriaceae* (Carattoli *et al.*, 2005). The main incompatibility groups in *Enterobacteriaceae* are HI1, HI2, I1, X, L/M, N, FIA, FIB, FIC, W, Y, P, A/C, T, K, and B/O (Carattoli, 2009). Among these, the ones mainly associated with antibiotic resistance genes are F, A/C, L/M, I1, HI2, and N.

Plasmid-mediated 16S rRNA MTs are usually borne by plasmids belonging to the IncA/C, IncN, IncL/M, and IncF groups (Table 10). IncA/C plasmids have been thoroughly studied in the last 10 years, since they have been found to be the primary plasmids responsible for the spread of the bla_{CMY-2} cephalosporinase gene prevalent among *E. coli* and *Salmonella* spp. isolates in North America and Europe (Carattoli *et al.*, 2012). They are broad host range plasmids, therefore they can replicate in *Enterobacteriaceae* but also in *Photobacterium damselae* and *Aeromonas salmonicida*. IncA/C-type plasmids have been detected in strains from humans as well as from animals. IncN plasmids have mainly been associated with strains of animal origins, additionally they are involved in the transmission of the $bla_{CTX-M-1}$ gene, which suggests an animal reservoir for this ESBL. These plasmids are highly prevalent in *E. coli* from the avian faecal flora and in *Salmonella* spp. from retail meat and food-producing animals (Johnson *et al.*, 2007). Plasmids belonging to the IncL/M family are broad-host-range and have been associated with the spread of $bla_{CTX-M-3}$ among human strains in Poland, pCTX-M-3 being the representative plasmid of this family (Baraniak *et al.*, 2002). The IncF-type plasmids are low-copy-number plasmids limited to the *Enterobacteriaceae* by their host-range. They are not a homogeneous group, presenting a large variation in size (from 50 to 200 kb), and they often carry more than one replicon, as they can carry the repFII replicon alone or in combination with repFIA and/or repFIB. These IncF plasmids have widely been associated with the dissemination of the pandemic CTX-M-15 β-lactamase (Woodford, 2008).

The *armA* gene was found to be located on IncA/C and IncHI2 plasmids in Asian strains isolated before 2001, but later on it became prevalently linked to IncF, IncL/M, and untypeable plasmids (Kang *et al.*, 2008). It was suggested that this plasmid transition was responsible for the emergence of multidrug-resistant *K. pneumoniae* in these countries (Carattoli, 2009). At that time, IncL/M plasmids were associated with the dissemination of the *armA* gene in clinical isolates from European countries, pCTX-M-3 specifically was considered to be responsible for the spread of this acquired MT in Poland (Zacharczuk *et al.*, 2011). However, it is important to highlight that *armA* was located on an IncN plasmid from an animal

origin in Spain (Gonzalez-Zorn *et al.*, 2005a), and more recently, the emergence of NDM-1-positive IncA/C plasmids bearing *armA* has been described.

Table 10. Main plasmid incompatibility types found to bear a MT gene[a]

Acquired 16S rRNA MT gene	Plasmid Incompatibility group
armA	**IncL/M** (*bla*$_{CTX-M-3}$[b], *bla*$_{CTX-M-15}$), **IncHI2**, **IncN**, **IncA/C** (*bla*$_{NDM-1}$), **IncF** (*bla*$_{KPC-2}$, *qnrB4*)
rmtA	**IncA/C** (*bla*$_{NDM-1}$)
rmtB	**IncI1**, **IncA/C**, **IncFI** (*qepA*), **IncFII** (*qepA*, *qnrS1*, *fosA3*, *bla*$_{CTX-M-15}$), **IncF49:A-:B-** (*qnrS1*, *bla*$_{CTX-M-15}$, *aac(6′)-Ib-cr*), **IncF33:A-:B-** (*bla*$_{CTX-M-9}$), **IncF2:A-:B-** (*qepA*)
rmtC	**IncA/C** (*bla*$_{NDM-1}$)
rmtD/D2	**IncA/C**
rmtE	ND[c]
rmtF	**Non-typable**
rmtG	**IncN**, **IncL/M**, **IncA/C**
rmtH	chromosomally located
npmA	ND

[a] Isolates from the publications constituting this Thesis are not taken into consideration
[b] Other important resistance determinants co-located on the same plasmid
[c] Not determined

Despite the *rmtB* gene having been shown to be located on different replicons such as IncI1 or IncA/C, the fully sequenced IncF plasmid pIP1206 from an *E. coli* isolated in France revealed the presence of both *rmtB* and *qepA* genes, the latter encoding a novel plasmid-mediated fluroquinolone efflux pump (Périchon *et al.*, 2008). Since then, *rmtB* has mostly spread out in China, linked to plasmids of the IncF family which have been further characterized following a typing scheme used to discriminate IncF variants (Villa *et al.*, 2010) (Table 10).

Most of these *rmtB* carriers are IncFII variants (Deng *et al.*, 2011b). For instance, F33:A-:B- and F2:A-:B- plasmids mediate the dissemination of *rmtB* associated with CTX-M-9 ESBLs and QepA genes, respectively (Deng *et al.*, 2011a). Regarding the rest of the acquired MT genes, IncA/C may be the main incompatibility group found in association to these genes (Table 10).

3.3.2- Mobilization platforms and close genetic environment of acquired 16S rRNA MTs

The genetic environment of 16S rRNA MT genes has been elucidated in some cases. Deciphering these environments is crucial because it will contribute to the understanding of how genetic elements involved in the mobilization of these genes accelerate their spread. Furthermore, it will aid in the prediction of the evolutionary pathways of these resistance determinants. Below, the genetic regions clarified so far are outlined, and the mobile elements associated with each MT gene are summarized in Table 11.

- *armA.* Although it is the most prevalent acquired MT to date, its genetic context has only been elucidated in certain strains. All *armA* surroundings analyzed to date share a high identity. Usually *armA* is found flanked by two transposases taking part in the Tn*1548* composite transposon, which comprises amongst others, a class 1 integron and an IS*CR1* element upstream of *armA*. After the earliest identification of ArmA, its gene was shown to be disseminating among different human strains in Europe in the same Tn*1548* structure as the pCTX-M-3 plasmid (Galimand *et al.*, 2005; Golebiewski *et al.*, 2007). However, the complete sequence of plasmid pMUR050, from an animal isolate in Spain (Gonzalez-Zorn *et al.*, 2005a), revealed that *armA* was located on a Tn*1548* lacking a *dhfrXII* trimethoprim resistance cassette. Recently, a few other *armA*-containing regions have been published (Du *et al.*, 2010; Jiang *et al.*, 2010; Ho *et al.*, 2011), and all of them are related to Tn*1548*.

- *rmtA.* The genetic environment of *rmtA* has been elucidated in different strains of *P. aeruginosa* (Yamane *et al.*, 2004), and although not identical, *rmtA* is embedded

in a region that shares similarity with the composite transposon Tn*5041*, previously described to be involved in mercury resistance in *Pseudomonas* spp. In addition, *rmtA* is bracketed by two copies of a kappa-gamma (κγ) mobile element previously shown to belong to the Tn*3* family of transposons, known for containing several transposase and resolvase genes. Therefore, it was hypothesized that after the first insertion of *rmtA* into a Tn*5041*, the latter was responsible for its spread among *Pseudomonas* spp. strains (Yamane *et al.*, 2004).

- *rmtB.* This MT gene has generally been found on fragments flanked by two IS*26* insertion sequences, associated with Tn*3* transposon structures and IS*CR3* elements. It is frequently accompanied by a Tn*3*-like structure containing an upstream $bla_{\text{TEM-1}}$, as well as the downstream fluoroquinolone efflux transporter gene, *qepA* (Perichon *et al.*, 2008; Li *et al.*, 2012).

- *rmtC.* When it had only been found in *P. mirabilis*, the presence of an IS*Ecp1* element at the 5' end of *rmtC* was deciphered. This element was shown to contain a transposase gene responsible for the transposition of *rmtC*, as well as to promote its expression (Wachino *et al.*, 2006a). When this MT was discovered in *S. enterica* isolates from the UK, the presence of an IS*Ecp1* immediately upstream of *rmtC* was found when amplifying a DNA fragment from *rmtC* to the end of IS*Ecp1* by using the primers previously described (Zong *et al.*, 2008). However, the complete IS*Ecp1* element could not be amplified by using internal primers, indicating either a partial deletion or the involvement of a different IS*Ecp1*-like element in the dissemination of *rmtC* in *Salmonella* (Hopkins *et al.*, 2010). At the same time, the report of RmtC in *Salmonella* isolates from the USA identified the 3´end of the IS*Ecp1* along with one inverted repeat region upstream of *rmtC*, but its whole sequence remains uncertain (Folster *et al.*, 2009).

- *rmtD/D2.* The genetic environment of *rmtD* was investigated in *P. aeruginosa* and *K. pneumoniae* strains (Doi *et al.*, 2008a). In both cases the *rmtD* gene is bound by 2 copies of an IS*CR14* element in the same orientation, and a downstream class 1 integron follows *rmtD*. In *K. pneumoniae*, both copies of IS*CR14* are truncated with an IS*26* element, thus *rmtD* appears to have been

mobilized from *P. aeruginosa* by an IS*26*-mediated event. The genetic surrounding of *rmtD2* was similar to that of *rmtD* in *P. aeruginosa* (Tijet *et al.*, 2011).

Table 11. Genetic elements potentially involved in the mobilization of the 16S rRNA MT genes.

Acquired 16S rRNA MT gene	Mobile genetic element
armA	Tn*1548*, IS*26*
rmtA	Tn*5041*
rmtB	IS*26*, Tn*3*, IS*CR3*
rmtC	IS*Ecp1*
rmtD/D2	IS*CR14*, IS*26*
rmtE	ND[a]
rmtF	*insE*
rmtG	ND
rmtH	IS*CR2*
npmA	IS*26*

[a] Not determined

- *rmtF.* Its close environment consists of an *insE* transposase downstream of *rmtF* followed by an *oriIS* duplicated immediately upstream of *rmtF*. There were also an aminoglycoside resistance *aac(6')-Ib* and a chloramphenicol resistance *cat* gene within the cloned fragment containing *rmtF*.

- *rmtH.* In its first and only description, *rmtH* is located in the chromosome and bracketed by two copies of IS*CR2* (O´Hara *et al.*, 2013). IS*CR2* is an IS*91*-like transposable element that has been found in association with several resistance genes and it is considered to facilitate the mobilization of downstream genetic elements (Toleman *et al.*, 2006).

- *npmA.* In its first and only identification, this gene was located on a 9.1 kb fragment enclosed by two IS*26* elements in the same orientation. This region does not share similarity with any other sequence in the database, but its vicinity shows a significant likeness with that of different multidrug-resistance plasmids sequences deposited in GenBank (Wachino *et al.*, 2007).

- *rmtE, rmtG.* The genetic context of these two genes has not yet been elucidated.

3.4- Association of the acquired 16S rRNA MTs with other antibiotic resistance mechanisms

The emergence of acquired 16S rRNA MTs in gram-negative bacteria is of a great concern because aminoglycosides are still useful antibiotics for the treatment of several infections due to both gram-negative and gram-positive bacteria, but also, their use has been strongly encouraged as an alternative for those infections caused by bacteria resistant to other antibiotics (Livermore *et al.*, 2011a). As previously described, acquired 16S rRNA MT genes are usually found within large conjugative plasmids, which possess the ability of accumulating various antibiotic resistance genes. Thus, acquired 16S rRNA MTs-producing bacteria are known to potentially develop multidrug resistance, their association to quinolone and β-lactam resistance being the most worrisome. Furthermore, they can either be co-produced by the same bacteria, or linked to the same genetic structure.

- *Association with quinolone resistance determinants*

Quinolones are bactericidal agents widely used in human and veterinary medicine. They are divided into first generation quinolones (such as nalidixic acid), and second generation quinolones also called fluoroquinolones (such as norfloxacin or ciprofloxacin). Both groups show a high activity agains gram-negative bacteria, and fluoroquinolones were developed to exhibit an increased action towards gram-positive bacteria (Jacoby, 2005). Besides the largely known quinolone resistance mechanism, which is chromosomally encoded and functions by mutating the antibiotic target site (DNA gyrase and Topoisomerase IV), in the

last decade the emergence of plasmid-mediated quinolone resistance (PMQR) further raised the significance of antibiotic resistance (Poirel *et al.*, 2012). The PMQRs discovered so far are Qnr, which protects the DNA gyrase and Topoisomerase IV from quinolone binding; the AAC(6')-Ib-cr, an aminoglycoside modifying enzyme variant able to acetylate ciprofloxacin; and the efflux pump QepA, that causes a significant decrease in susceptibility to the hydrophilic quinolones (i.e., ciprofloxacin, enrofloxacin).

PMQR has been associated with acquired 16S rRNA MTs in strains producing ArmA, RmtB, or RmtC. The latter has only been found in association with PMQR in two *K. pneumoniae* strains recently isolated from Bangladesh, which co-produce QnrB (Islam *et al.*, 2012). ArmA has only been reported in association with PMQR a few times despite its high prevalence, most of which were in China (Jiang *et al.*, 2010; Du *et al.*, 2012; Guo *et al.*, 2012; McGann *et al.*, 2012). It has been found to be co-produced with QnrS1, QnrB4, QnrA, QnrB2, and the enzyme AAC(6')-Ib-cr, the last two of which were detected in chicken isolates. On the other hand, most RmtB reports are associated with the emergent efflux pump QepA, initially detected during a screening for aminoglycoside resistance MTs (Perichon *et al.*, 2007). In addition, RmtB has been found with the *aac(6')-Ib-cr* variant as well as with several *qnr* genes of the A, B, and C variants. Moreover, in some cases the co-expression of several PMQR determinants coupled with RmtB has been observed (Liu *et al.*, 2008). Most of the publications concerning RmtB linked to PMQR arise from bacteria isolated from animals (Liu *et al.*, 2008; Deng *et al.*, 2011a, 2011b).

- *Association with β-lactam resistance determinants*

Given that β-lactams are one of the main families of antibiotics classified by the World Health Organization as critically important for the human medicine, the acquisition of β-lactam resistance determinants by gram-negative bacteria producing 16S rRNA MTs is a great cause for alarm. Besides the narrow and broad spectrum β-lactamases, such as a number of TEM and SHV enzymes as a resistance mechanism to penicillins, and first and second generation cephalosporins, the emergence and increase of ESBLs (Extended-spectrum β-

lactamases), acquired AmpC enzymes, and carbapenemases in the last years pose a major concern regarding antibiotic resistance. In fact, it has been suggested that co-selection with other resistance mechanisms (i.e., fluoroquinolones or aminoglycosides) may have contributed to this issue (Coque *et al.*, 2008).

Regardless of the variety of existing ESBLs, since their first identification the acquired 16S rRNA MTs have mainly been found in CTX-M-type producers (Table 12). Among these, the association with the globally prevalent CTX-M-15 variant has increasingly been reported in the last years (Table 15). But without a doubt, the emergence of carbapenem resistant bacteria in the last years and the recent findings on the coproduction of other resistance mechanisms such as the 16S rRNA MTs, are of a great concern. The main reason is that carbapenemases exhibit activity against a wide spectrum of β-lactams, including newer cephalosporins and carbapenems, which limits the few existing alternative options (i.e., aminoglycosides) for the treatment of these serious infections. Acquired 16S rRNA MTs have been found sporadically in bacteria producing SPM-1, IMP, and VIM metallo-β-lactamases (MBLs) (Doi *et al.*, 2007c; Lee *et al.*, 2007; Gurung *et al.*, 2010; Livermore *et al.*, 2011a). Most worrisome is the increasing number of reports of 16S rRNA MTs among OXA-type and KPC-type carbapenemases in the last years. Both ArmA and RmtB have repeteadly been identified in association with KPC-type carbapenemases, of great overall relevance in *K. pneumoniae* infections (Jiang *et al.*, 2010; Sheng *et al.*, 2012), whereas an emergence of ArmA in OXA-type producing *A. baumanii* worldwide has been observed, with an increased incidence in Asian countries (Doi *et al.*, 2007b; Kumarasamy *et al.*, 2010; Karah *et al.*, 2011; Livermore *et al.*, 2011a). In addition, NDM-1 (New Delhi Metalo β-lactamase-1), firstly reported in 2009, has become a major global health issue due to its rapid spread and resistance spectrum (Kumarasamy *et al.*, 2010). Nowadays, the existence of NDM-producers with 16S rRNA MTs has already been reported. Furthermore, this linkage is usually associated to large multiresistant conjugative plasmids, and it appears to be a growing problem worldwide (Livermore *et al.*, 2011b; Solé *et al.*, 2011; Dortet *et al.*, 2012; Dolejska *et al.*, 2013).

Table 12. Coproduction of acquired 16S rRNA MTs and ESBLs.

Bacteria	MT genes	ESBL genes	Country	Year of Publication	Reference
K. pneumoniae	*armA*	$bla_{CTX\text{-}M\text{-}3}$	France	2003	Galimand *et al.*
E. coli, K. pneumoniae	*armA*	$bla_{CTX\text{-}M\text{-}3}$, $bla_{CTX\text{-}M\text{-}14}$, $bla_{SHV\text{-}5}$	Taiwan	2004	Yan *et al.*
Enterobacteriaceae	*armA, rmtB*	$bla_{CTX\text{-}M\text{-}3}$, $bla_{CTX\text{-}M\text{-}9}$, $bla_{CTX\text{-}M\text{-}14}$, $bla_{SHV\text{-}12}$	Korea	2006	Park *et al.*
Enterobacteriaceae	*armA, rmtB*	$bla_{CTX\text{-}M\text{-}3}$, $bla_{CTX\text{-}M\text{-}14}$	Belgium	2007	Bogaerts *et al.*
Enterobacteriaceae	*armA*	$bla_{CTX\text{-}M\text{-}3}$	Poland	2007	Golebiewski *et al.*
Providencia spp.	*armA*	$bla_{PER\text{-}1}$	Korea	2007	Lee *et al.*
Proteus mirabilis	*rmtC*	$bla_{VEB\text{-}6}$	Australia	2008	Zong *et al.*
P. mirabilis	*armA, rmtB*	$bla_{CTX\text{-}M\text{-}3}$, $bla_{CTX\text{-}M\text{-}4}$	Taiwan	2008	Wu *et al.*
Enterobacteriaceae	*armA*	$bla_{CTX\text{-}M\text{-}3}$	Bulgaria	2008	Sabtcheva *et al.*
K. oxytoca	*armA*	$bla_{CTX\text{-}M\text{-}15}$	China	2008	Zhang *et al.*
Enterobacteriaceae	*armA, rmtB*	$bla_{CTX\text{-}M\text{-}3}$, $bla_{CTX\text{-}M\text{-}14}$	Korea	2008	Kang *et al.*
A. baumanii	*armA*	$bla_{PER\text{-}1}$	Korea	2008	Kim *et al.*
Enterobacteriaceae	*armA, rmtB*	$bla_{CTX\text{-}M\text{-}3}$, $bla_{CTX\text{-}M\text{-}14}$	France	2008	Berçot *et al.*
E. coli	*armA, rmtB*	$bla_{CTX\text{-}M\text{-}14}$, $bla_{CTX\text{-}M\text{-}65}$	China	2009	Du *et al.*
K. pneumoniae	*armA*	$bla_{SHV\text{-}12}$	China	2009	Liu *et al.*

K. pneumoniae	*armA, rmtB*	$bla_{CTX-M-3}$, $bla_{CTX-M-14}$, $bla_{CTX-M-15}$, bla_{SHV-5}, bla_{SHV-12}	Taiwan	2009	Ma *et al.*
K. pneumoniae	*armA, rmtB*	$bla_{CTX-M-14}$, $bla_{CTX-M-15}$	China	2009	Yu F. *et al.*
E. coli	*rmtB*	$bla_{CTX-M-3}$, $bla_{CTX-M-14}$, $bla_{CTX-M-15}$, $bla_{CTX-M-24}$, $bla_{CTX-M-27}$	China	2010	Yu FY. *et al.*
K. pneumoniae	*rmtB*	$bla_{CTX-M-15}$	Turkey	2010	Berçot *et al.*
E. coli	*armA, rmtB*	$bla_{CTX-M-15}$	Australia	2010	Poirel *et al.*
Salmonella enterica	*armA*	$bla_{CTX-M-15}$	Algeria	2011	Bouzidi *et al.*
A. baumanii	*armA*	bla_{PER-7}	France	2011	Bonnin *et al.*
E. coli	*armA, rmtB*	$bla_{CTX-M-15}$	France	2011	Poirel *et al.*
E. coli	*armA*	$bla_{CTX-M-15}$	Spain	2011	Solé *et al.*
E. coli	*rmtB*	$bla_{CTX-M-15}$	USA	2011	Tian *et al.*
Enterobacteriaceae	*rmtB*	$bla_{CTX-M-9}$, $bla_{CTX-M-14}$, $bla_{CTX-M-27}$, $bla_{CTX-M-65}$	China	2011	Deng *et al.*
Enterobacteriaceae	*armA, rmtB*	$bla_{CTX-M-15}$, bla_{SHV-12}	Serbia, Pakistan	2011	Bogaerts *et al.*
S. enterica Gambia	*armA*	$bla_{CTX-M-3}$	France	2011	Moissenet *et al.*
S. enterica Infantis	*armA*	$bla_{CTX-M-15}$	Algeria	2001	Naas *et al.*
K. pneumoniae	*armA*	$bla_{CTX-M-3}$	Sweden	2011	Samuelsen *et al.*
K. pneumoniae	*armA*	bla_{CTX-M}	Poland	2011	Zacharczuk *et al.*
E. coli	*armA, rmtB*	$bla_{CTX-M-15}$	Bangladesh	2011	Poirel *et al.*
K. pneumoniae	*armA*	$bla_{CTX-M-14}$	China	2011	Lee *et al.*
K. pneumoniae	*rmtC*	$bla_{CTX-M-15}$	Kenya, India	2011	Poirel *et al.*
Enterobacteriaceae	*rmtD2*	bla_{CTX-M}	Argentina	2011	Tijet *et al.*

S. enterica Paratyphi B	*armA*	$bla_{CTX-M-3}$	China	2012	Du *et al.*
E. coli	*rmtB*	$bla_{CTX-M-3}$, $bla_{CTX-M-14}$, $bla_{CTX-M-27}$, $bla_{CTX-M-55}$, $bla_{CTX-M-65}$	China	2012	Hou *et al.*
E. coli	*rmtB*	$bla_{CTX-M-15}$	China	2012	Li *et al.*
K. pneumoniae, Enterobacter amnigenus	*rmtB*	$bla_{CTX-M-3}$, $bla_{CTX-M-14}$, bla_{SHV-12}	China	2012	Sheng *et al.*
Enterobacteriaceae	*rmtC*	$bla_{CTX-M-15}$	New Zealand	2012	Williamson *et al.*
K. pneumoniae	*rmtH*	$bla_{CTX-M-15}$	Iraq	2013	O´Hara *et al.*
K. pneumoniae	*rmtD, rmtG*	$bla_{CTX-M-2}$, $bla_{CTX-M-15}$, $bla_{CTX-M-59}$	Brazil	2013	Bueno *et al.*

- *Association with other resistance determinants*.

Among the acquired 16S rRNA MTs producers, multiple determinants conferring resistance to different antibiotic classes can be found. Other than those mechanisms encoded by genes that belong to the bacterial chromosome (i.e., resistance to rifampicin by target mutation), most of the resistance genes linked to the 16S rRNA MTs are located on plasmids or mobile elements. Hence, genetic platforms such as integrons facilitate the capture of several resistance genes in a single cassette. One example could be the Tn*1548* in which *armA* is embedded; besides the macrolide resistance genes located downstream of *armA*, this transposon contains one class 1 integron with various resistance genes to sulfonamides, trimethoprim, or aminoglycosides (Gonzalez-Zorn *et al.*, 2005a). Apart from the β-lactam and quinolone resistance genes previously described, it is common to detect the presence of genes conferring resistance to tetracycline (i.e., *tet*), macrolides (i.e., *mel*, *mph2*), chloramphenicol (i.e., *cat*), or even multiple

aminoglycoside modifying enzymes in 16S rRNA MTs produing-bacteria (Jiang *et al.*, 2010; Sekizuka *et al.*, 2011; Wachino and Arakawa, 2012; Dolejska *et al.*, 2013).

OBJECTIVES AND JUSTIFICATION

In the last decades, the rapidly growing emergence and spread of bacterial resistance to antibiotics, combined with a decreasing discovery of new effective molecules to combat bacterial infections, has turned antibiotic resistance into one of the major concerns for Public Health. Bacterial infections caused by MDR gram-negative bacilli have become one of the biggest obstacles in both hospitals and the community. As a matter of fact, 4 out of the 6 famous ESKAPE pathogens (described in the introduction section and recognized as one of the main threats of this century) are gram-negative bacilli.

Aminoglycosides are considered broad-spectrum antimicrobials that exhibit bactericidal activity against both gram-positive and gram-negative infections. This family of antibiotics is widely used in human and veterinary medicine, and they are still one of the most valuable weapons to combat infections caused by gram-negative pathogens. They often constitute the treatment of choice for infections due to MDR strains. The recent emergence of the 16S rRNA methyltransferases in gram-negative bacteria, poses a serious problem regarding the efficacy of all clinically available aminoglycosides.

The objectives of this Doctoral Thesis are the identification and molecular characterization of aminoglycoside resistance methyltransferases in gram-negative bacteria from humans, animals, and food. This will contribute to a better understanding of the role that animals and food play in the transmission of these resistance determinants. Furthermore, it will lead to a deeper knowledge of the epidemiology and the genetic platforms involved in the mobilization of these genes. The monitoring and study of emerging resistance mechanisms is crucial if antibiotic resistance is to be delayed.

PUBLICATIONS

This Doctoral Thesis is presented in publications format, and it consists of four works. Three of these works have been published in the journal *Antimicrobial Agents and Chemotherapy* (ISI 2012, Impact Factor: 4,565), and one of the works has been published in the *Journal of Antimicrobial Chemotherapy* (ISI 2012, Impact Factor: 5,338).

1) ArmA methyltransferase in a monophasic *Salmonella enterica* isolate from food

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ArmA Methyltransferase in a Monophasic *Salmonella enterica* Isolate from Food[▽]

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The 16S rRNA methyltransferase ArmA is a worldwide emerging determinant that confers high-level resistance to most clinically relevant aminoglycosides. We report here the identification and characterization of a multidrug-resistant *Salmonella enterica* subspecies I.4,12:i:− isolate recovered from chicken meat sampled in a supermarket on February 2009 in La Reunion, a French island in the Indian Ocean. Susceptibility testing showed an unusually high-level resistance to gentamicin, as well as to ampicillin, expanded-spectrum cephalosporins and amoxicillin-clavulanate. Molecular analysis of the 16S rRNA methyltransferases revealed presence of the *armA* gene, together with bla_{TEM-1}, bla_{CMY-2}, and $bla_{CTX-M-3}$. All of these genes could be transferred *en bloc* through conjugation into *Escherichia coli* at a frequency of 10^{-5} CFU/donor. Replicon typing and S1 pulsed-field gel electrophoresis revealed that the *armA* gene was borne on an ~150-kb broad-host-range IncP plasmid, pB1010. To elucidate how *armA* had integrated in pB1010, a PCR mapping strategy was developed for Tn*1548*, the genetic platform for *armA*. The gene was embedded in a Tn*1548*-like structure, albeit with a deletion of the macrolide resistance genes, and an IS*26* was inserted within the *mel* gene. To our knowledge, this is the first report of ArmA methyltransferase in food, showing a novel route of transmission for this resistance determinant. Further surveillance in food-borne bacteria will be crucial to determine the role of food in the spread of 16S rRNA methyltransferase genes worldwide.

Aminoglycosides are used for the treatment of a wide range of infections due to both Gram-negative and Gram-positive bacteria. Resistance to these antimicrobials is usually due to the production of aminoglycoside-modifying enzymes, which are able to acetylate, phosphorylate, or adenylate the antibiotic molecule (8). Since 2003, the 16S rRNA methyltransferases have emerged in Gram-negative pathogens as an acquired aminoglycoside resistance mechanism that confers high-level resistance to most clinically relevant aminoglycosides (13, 17). Seven different 16S rRNA methyltransferase genes have been described thus far: *armA, rmtA, rmtB, rmtC, rmtD, rmtE*, and *npmA* (9, 10, 35). *armA* and *rmtB* are the most prevalent among *Enterobacteriaceae*. Except for the identification of *armA* and *rmtB* in animal isolates from Spain and China (5, 11, 17) and *rmtC* in *Salmonella enterica* from food in the United Kingdom (23), all methyltransferase genes described to date were identified in human clinical samples (36). Although *rmtC* has been reported in the bacterial chromosome integrated through the IS*Ecp*1 mobile element (23), in most cases these genes have been related to large conjugative plasmids in association with extended-spectrum β-lactamases (ESBLs) or, more recently, carbapenemases such as KPC-2 (25) or NDM-1 (29). Although the genetic plasticity of these conjugative elements is high, *armA* has been always identified in the transposable element Tn*1548* (14, 16).

In order to determine whether food animal products are a source of 16S rRNA methyltransferases, we studied the presence of these genes in bacteria isolated from food in La Reunion Island. We report here a multidrug-resistant *Salmonella enterica* isolate, 09CEB904SAL, recovered from food and producing the ArmA methyltransferase. The isolate was identified as *S. enterica* I.4,12:i:−, a monophasic variant of *S. enterica* serovar Typhimurium. Molecular characterization of 09CEB904SAL revealed the presence of *Salmonella* genomic island 1 (SGI1) (2, 19, 27) and of the virulence plasmid *S.* Typhimurium pSLT (31). In addition, three β-lactamase genes—bla_{TEM-1}, bla_{CMY-2}, and $bla_{CTX-M-3}$—were detected, the latter borne on a broad-host-range IncP conjugative plasmid together with the *armA* methyltransferase gene. To our knowledge, this is the first report of ArmA methyltransferase from food. This reflects a novel transmission route for *armA* and confirms presence of 16S rRNA methyltransferases in the East of Africa.

(An initial report of this study was presented in the 49th Interscience Conference on Antimicrobial Agents and Chemotherapy in September 2009 in San Francisco, CA.)

MATERIALS AND METHODS

Bacterial strains and antimicrobial susceptibility testing. 09CEB904SAL, an *S. enterica* isolate, was recovered from chicken meat sampled during a control by the retailer in a supermarket on February 2009 in La Reunion, a French island in the Indian Ocean (22). The isolate was serotyped on the basis of the somatic O antigen and phase 1 and phase 2 flagellar H antigens, as specified by the White-Kauffmann-Le Minor scheme (18) using agglutination tests with antisera

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TABLE 1. Primers used in this study

Primer	Sequence (5′–3′)	Position[a] or amplicon size (bp)	Source or reference
Tn1F	GGCACTGTTGCAAATAGTCGG	10326–10346	This study
Tn1R	TTGCTGCTTGGATGCCCGAGG	11981–12001	This study
Tn2F	CCGGGTGACGCACACCGTGGA	11784–11804	This study
Tn2R	TCATTTACCAACTGACTTGAT	12863–12883	This study
Tn3F	TTATTCGCTTTGTGAAAGGCG	12837–12857	This study
Tn3R	TCCAGACGGCCACATTGGAGG	14817–14837	This study
Tn4F	TCAACGACCTCCTCCAATGTG	14807–14827	This study
Tn4R	ACCGCATGGGTTGTGGCATCC	16787–16807	This study
Tn5F	GGATGCCACAACCCATGCGGT	16787–16807	This study
Tn5R	ACAATAAGATTGTTGTACTTT	18813–18833	This study
Tn6F	TATGGGCAGGGCGAAGCGCTA	18792–18812	This study
Tn6R	TTTGAGTAAACTACTCTTTCC	20773–20793	This study
Tn7F	CGTATTGGTCTTGTGGGTGAT	20743–20763	This study
Tn7R	GTGAAATCTGCCCATAGAACA	22722–22742	This study
Tn8F	GGGAAAATGATTTGAAAATTA	22589–22609	This study
Tn8R	CTGCTATTGTGCCTTTAATTA	24589–24609	This study
Tn9F	TTGTTCTTCTAACCTAGTAAT	24572–24592	This study
Tn9R	CACTGTTGCAAAGTTAGCGAT	25584–25604	This study
armAF	CAAATGGATAAGAATGATGTT	774	13
armAR	TTATTTCTGAAATCCACT	774	13
rmtA.F	ATGAGCTTTGACGATGCCCTA	756	This study
rmtA.R	TCACTTATTCCTTTTTATCATG	756	This study
rmtB.F	ATGAACATCAACGATGCCCT	769	36
rmtB.R	CCTTCTGATTGGCTTATCCA	769	36
rmtC.F	CGAAGAAGTAACAGCCAAAG	711	10
rmtC.R	ATCCCAACATCTCTCCCACT	711	10
rmtD.F	CGGCACGCGATTGGGAAGC	401	10
rmtD.R	CGGAAACGATGCGACGAT	401	10
rmtE.F	ATGAATATTGATGAAATGGTTGC	818	9
rmtE.R	TGATTGATTTCCTCCGTTTTTG	818	9
U7-L12	ACACCTTGAGCAGGGCAAAG	SGI1 left junction	2
LJ-R1	AGTTCTAAAGGTTCGTAGTCG	SGI1 left junction	2
104-RJ	TGACGAGCTGAAGCGAATTG	SGI1 right junction	2
C9L2	AGCAAGTGTGCGTAATTT	SGI1 right junction	2
L1	GGCATCCAAGCAGCAAG	5′CS of class 1 integron	26
R1	AAGCAGACTTGACCTGA	3′CS of class 1 integron	26

[a] That is, the position in plasmid pMUR050 (AY522431).

(obtained from Bio-Rad [Marnes-la-Coquette, France] and AES [Bruz, France]) according to an in-house method certified by the French Accreditation Committee (accreditation no. 1-0245). The absence of flagellar phase 2 antigens was confirmed following phase inversion method as recommended by EFSA (12). *Escherichia coli* K802N was used as the recipient strain for plasmid conjugation experiments. Antimicrobial susceptibility tests were first performed by disk diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLSI) (6). MICs were performed, and results were interpreted according to CLSI guidelines (7).

DNA analysis and manipulation. ESBL genes were detected by using Check KPC ESBL (Check-Points B.V., Wageningen, The Netherlands), a microarray using Clondiag technology (Jena, Germany) capable of detecting bla_{KPC} and groups of bla_{CTX-M} and discriminate ESBLs from non-ESBLs for bla_{TEM} and bla_{SHV}.

SGI1 was detected by amplifying the right and left junction and the class1 integron gene cassette as previously described (1, 26). The bla_{CMY-2} gene was detected as previously described (28). PCRs for all of the methylase genes were performed with the primers listed in Table 1. Plasmid DNA extraction was performed using a Plasmid Midi kit (Qiagen, Inc., Chatworth, CA). Tn*1548* mapping was carried out using plasmid extractions as a template and by performing overlapping PCRs with nine pairs of designed primers along the transposon (Table 1 and Fig. 2). The PCR products were purified and subsequently sequenced with their corresponding primers.

Conjugation experiments. Conjugation experiments were carried out in brain heart infusion (BHI) broth with 09CEB904SAL as the donor and *E. coli* K802N (which is resistant to nalidixic acid) as the recipient. Both strains were grown separately in BHI with moderate shaking until the donor strain reached an optical density at 600 nm of 0.9. Then, 1 ml of the donor culture, 1 ml of the recipient culture, and 1 ml of fresh BHI were mixed in a sterile flask, followed by incubation for 2 to 3 h at 37°C without shaking. Transconjugants were selected on BHI agar plates with nalidixic acid (50 μg/ml) and either ampicillin or gentamicin (50 μg/ml) to select for plasmid-encoded aminoglycosides and β-lactam resistance. Putative transconjugants were confirmed not to be nalidixic acid-resistant mutant donors by antimicrobial susceptibility testing and subsequent comparison with the donor and recipient resistance phenotype. *armA* PCR and S1 pulsed-field gel electrophoresis (S1-PFGE) were performed with the transconjugants. The conjugation frequency for each plasmid was calculated as the percentage of grown transconjugants bearing each plasmid versus the total conjugation frequency of the donors. To assess the possibility for the conjugation of more than one plasmid, 100 colonies grown using ampicillin-selected conjugation were replicated onto selective plates of gentamicin and amoxicillin-clavulanate in order to determine transconjugants with a plasmid bearing *armA,* bla_{CMY-2}, or both genes.

Plasmid analysis. In order to determine plasmid size, agarose gel plugs of total DNA were prepared according to the PulseNet PFGE protocol for the subtyping of *E. coli* O157:H7, *Salmonella* spp., and *Shigella* spp. (30). These plugs were digested with S1 nuclease (Promega, Madison, WI) according to the manufacturer's instructions. Plugs were loaded into a 1% agarose gel. PFGE was performed as previously described (32) with the following modifications: a running time of 21 h, a temperature of 14°C, a field strength of 6 V/cm, an included angle of 120°, an initial pulse time of 2.2 s, and a final pulse time of 63.8 s. The gels were stained with Sybr Safe (Invitrogen, Paisley, United Kingdom) for 20 min, destained in MilliQ water, and photographed under UV light. Lambda-ladder PFGE marker (New England Biolabs, Ipswich, MA) was used for molecular weight determinations. A PCR method based on five multiplex and three simplex PCRs was performed in order to determine the plasmid incompatibility group (4).

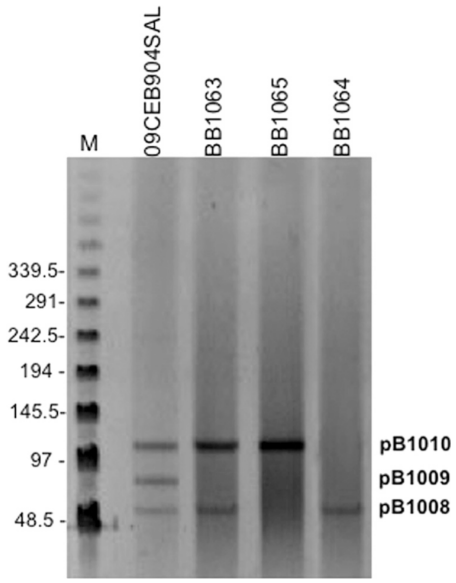

FIG. 1. Plasmid profile of the strains used in the present study. M, lambda ladder molecular marker. The numbers on the left show molecular weights in kilobase pairs. *Salmonella enterica* 09CEB904SAL and transconjugants BB1063, BB1065, and BB1064 are indicated in lanes 2 to 5, respectively. Each band represents one plasmid. *S. enterica* 09CEB904SAL bears pB1008, pB1009, and pB1010. The image reveals three types of transconjugants, one with pB1008 alone, one with pB1010 alone, and one with both pB1008 and pB1010.

Nucleotide sequence accession number. A partial sequence of the Tn*1548* variant observed in the present study has been deposited in GenBank under accession number HQ728525.

RESULTS AND DISCUSSION

Identification and characterization of *S. enterica* 09CEB904SAL. In February 2009, microbial analyses of food isolates in supermarkets from La Reunion Island identified a chicken meat sample with the presence of *S. enterica* 09CEB904SAL. This *Salmonella* strain was serotyped as *S. enterica* subspecies I.4,12:i:−. A complete antimicrobial profile showed that the bacterium possessed an unusual high-level multidrug resistance profile to ampicillin, amoxicillin-clavulanate, cephalothin, cefotaxime, ceftazidime, cefoxitin, streptomycin, kanamycin, gentamicin, sulfonamides, trimethoprim, tetracycline, and chloramphenicol (Table 2).

PCR for the SGI1 showed that this isolate bore the canonical right and left junctions from *S.* Typhimurium (Table 1). Integron cassette analysis identified two genes cassettes of 1,000 and 1,200 bp, which have been previously described in DT104 *S.* Typhimurium (2). PFGE analysis, performed as previously described (24), showed only 71% similarity with the subspecies I.4,12:i:− profile of the European epidemic clone STYMXB.0131 (24).

Identification and characterization of resistance determinants. Conjugation experiments were performed using 09CEB904SAL as the donor, *E. coli* K802N as the recipient, and gentamicin, ampicillin, and nalidixic acid as the selection markers. Analysis of the transconjugants revealed presence of two different phenotypes corresponding with two types of transconjugants: (i) transconjugant BB1063 expressing high-level resistance to gentamicin, ampicillin, amoxicillin-clavulanate, cefotaxime, ceftazidime, cefoxitin, and sulfonamides (Table 2) and (ii) transconjugant BB1064 showing only high-level resistance to ampicillin, amoxicillin-clavulanate, and ceftazidime but not to aminoglycosides or sulfonamides. In order to study the high-level resistance to aminoglycosides and beta-lactams, primers targeting all of the 16S rRNA methyltransferase genes, as well as the main ESBL genes, were used with plasmid extractions of the wild-type bacterium and transconjugants as a template (Table 1). KPC ESBL microarray and conventional PCR and sequencing (21) confirmed the presence of a $bla_{\text{CTX-M-3}}$ gene and a non-ESBL $bla_{\text{TEM-1}}$ gene. DNA fragments corresponding to *armA,* $bla_{\text{CMY-2}}$, $bla_{\text{TEM-1}}$, and group 1 $bla_{\text{CTX-M-3}}$ were amplified from the wild-type strain and sequenced. Concordantly, a non-ESBL $bla_{\text{TEM-1}}$ and a group 1 $bla_{\text{CTX-M-3}}$ were identified from the wild-type and transconjugant BB1063, whereas only $bla_{\text{CMY-2}}$ was amplified from transconjugant BB1064, suggesting the presence of two different plasmids in 09CEB904SAL. To the best of our knowledge, this is the first report of *armA* gene in an isolate from food origin. Previously, *armA* has been identified from several Gram-negative pathogens as one of the most prevalent 16S rRNA methyltransferase genes (20, 37). To date, most 16S rRNA methyltransferase-producing Gram-negative bacteria were recovered from human clinical samples. However, in the case of *armA*, it has also been found in porcine and chicken *E. coli* isolates from Spain and China, respectively (11, 17).

Plasmid analysis. As mentioned before, conjugation experiments suggested that 09CEB904SAL bore at least two transferable plasmids. S1-PFGE analyses revealed the presence of three plasmids of approximately 50, 80, and 110 kb that were named pB1008, pB1009, and pB1010, respectively (Fig. 1). The plasmids were classified according to a PCR-based replicon typing protocol (4). The 110-kb plasmid (pB1010) belonged to the IncP incompatibility group, whereas the 50-kb (pB1008) and 80-kb (pB1009) plasmids were not typeable using this methodology. Interestingly, this is the first time that the *armA* gene has been documented on an IncP plasmid, despite the fact that IncP plasmids are broad-host-range plasmids, and they have been widely described since the 1980s among several genus of *Proteobacteria* generally associated with antibiotic resistance determinants (33, 34). This finding suggests that further spread of *armA* to other strains could be enhanced from this genetic platform, since genes linked to IncP plasmids have a large host range, including *Pseudomonas* spp. or even Gram-positive bacteria. To precisely calculate the conjugation frequency of plasmid pB1010 bearing *armA,* $bla_{\text{TEM-1}}$, and $bla_{\text{CTX-M-3}}$ and plasmid pB1008 bearing $bla_{\text{CMY-2}}$, a conjugation experiment using ampicillin was performed. Transconjugants were replicated onto gentamicin and amoxicillin-clavulanate plates, since resistance to the latter is due to $bla_{\text{CMY-2}}$. Plasmid pB1010 was transferred into *E. coli* at a frequency of 9×10^{-4} (giving rise to transconjugant BB1065), whereas pB1008 was transferred at 4×10^{-4} per donor CFU. The two plasmids were cotransferred at a frequency of 10^{-5} per donor CFU. No resistance determinant was identified on plasmid pB1009, but the *spvC* gene, which is characteristic of pSLT, the virulence plasmid of *S.* Typhimurium, was detected by PCR (3)

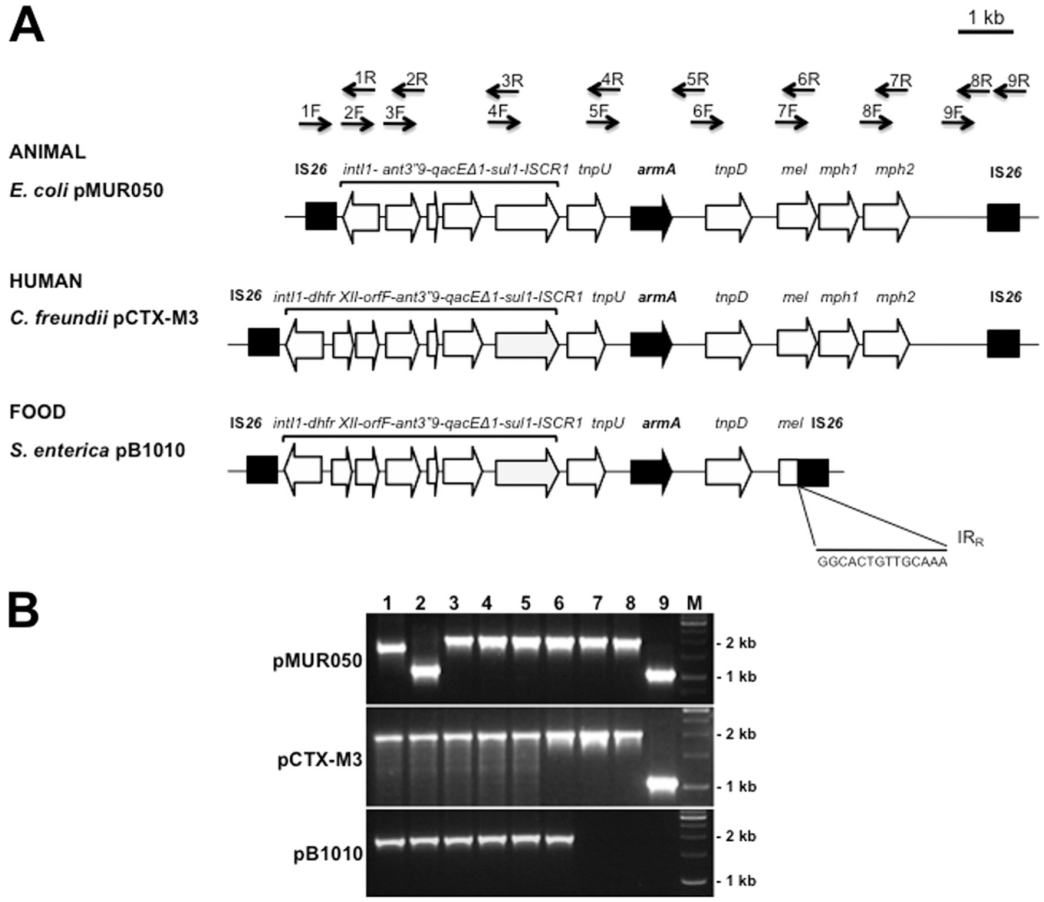

FIG. 2. (A) Genetic structure of Tn*1548* reported from an animal isolate (pMUR050), a human isolate (pCTX-M-3), and a food isolate of *Salmonella enterica* 09CEB904SAL (pB1010). Black arrows in the upper part of the panel represent pairs of primers designed for the mapping of Tn*1548*. (B) PCR fragments obtained in Tn*1548* mapping with plasmids from an animal isolate (pMUR050), a human isolate (pCTX-M-3), and a food isolate (pB1010). Lanes 1 to 9 represent the corresponding pairs of primers. M, molecular weight marker. The PCR results for lanes 7, 8, and 9 are negative in pB1010.

in 09CEB904SAL, with plasmids pB1008, pB1009, and pB1010, but not in the transconjugants (which lack pB1009), suggesting that pB1009 could be a pSLT derivative.

Mapping of Tn*1548*. Tn*1548* is the genetic platform that mobilizes *armA* between plasmids (14, 16). For this reason, a PCR mapping method for Tn*1548* was developed by designing nine pairs of primers that amplify overlapping PCR products. PCR fragments for the expected size were amplified from the control plasmids pCTX-M3 and pMUR050 (15, 17). In *Salmonella* 09CEB904SAL, Tn*1548* harbors the *dhfrXII* trimethoprim resistance cassette, like the strains bearing *armA* identified in clinical settings (13), whereas in the first *E. coli*

TABLE 2. MICs, resistance genes, and plasmids

Strain	MIC (mg/liter)[a]										Resistance gene(s)	Plasmid(s)
	AMC	AMP	CTX	CAZ	CIP	GEN	KAN	SXT	TET	TMP		
00CEB904SAL	>128	>32	>4	>16	0.06	>256	>128	>1,024	>64	>32	*armA*, $bla_{CTX-M-3}$, bla_{TEM-1}, bla_{CMY-2}	pB1008, pB1009, pB1010
K802N	2	4	0.12	0.5	0.25	0.5	≤4	≤8	≤1	≤0.5	None	None
BB1065	4	>32	>4	>16	0.25	>256	>128	>1,024	64	>32	*armA*, $bla_{CTX-M-3}$, bla_{TEM-1}	pB1010
BB1064	>128	>32	4	16	0.25	0.5	≤4	≤8	≤1	≤0.5	bla_{CMY-2}	pB1008
BB1063	>128	>32	>4	16	0.25	>256	>128	>1,024	>64	>32	*armA*, $bla_{CTX-M-3}$, bla_{TEM-1}, bla_{CMY-2}	pB1008, pB1010

[a] AMC, amoxicillin-clavulanate; AMP, ampicillin; CTX, cefotaxime; CAZ, ceftazidime; CIP, ciprofloxacin; GEN, gentamicin; KAN, kanamycin; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline; TMP, trimethoprim.

isolate described with *armA* and in animals, Tn*1548* lacked this cassette (16). Furthermore, in 09CEB904SAL, PCRs for the amplification of the downstream region of the transposon, corresponding to the pairs 7, 8, and 9 were negative. Suspecting the possibility of a deletion in that region, a long PCR with primers Tn6F and Tn9R was performed. An amplicon of ~3 kb was obtained, instead of the 7-kb amplicon that would correspond to the complete Tn*1548* element. Subsequent sequencing of this DNA fragment with the primers Tn6F and Tn9R revealed insertion of an IS*26* element with its IR_R region interrupting the macrolide resistance genes downstream *armA*. These data show that Tn*1548* is a dynamic element that is ultimately responsible for the spread of *armA*.

Overall, these results confirm the presence of *armA* in East Africa and imply a novel route of transmission for this emerging resistance determinant. Further surveillance in food-borne bacteria will be crucial to determine the role of food products in the spread of the 16S rRNA methyltransferase genes worldwide.

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2) Association of extended-spectrum β-lactamase VEB-5 and 16S rRNA methyltransferase ArmA in *Salmonella enterica* from the United Kingdom

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LETTER TO THE EDITOR

Association of Extended-Spectrum β-Lactamase VEB-5 and 16S rRNA Methyltransferase ArmA in *Salmonella enterica* from the United Kingdom

Aminoglycosides and β-lactams are used for the treatment of a wide range of infections due to both Gram-negative and Gram-positive bacteria. An emerging aminoglycoside resistance mechanism, methylation of the aminoacyl site of the 16S rRNA, confers high-level resistance to clinically important aminoglycosides such as amikacin, tobramycin, and gentamicin. Eight 16S rRNA methyltransferase genes, *armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD*, *rmtE*, *rmtF*, and *npmA*, have been identified in several species of enterobacteria worldwide (2, 6, 7, 9, 11, 13, 14). Resistance to extended-spectrum β-lactams remains, additionally, an important clinical problem. Apart from the large TEM, SHV, and CTX-M families, several other extended-spectrum β-lactamases (ESBLs) have been identified, including VEB enzymes, which confer high-level resistance to cephalosporins and monobactams. Although 16S rRNA methyltransferases have been frequently identified as associated with different ESBLs, there has been no report of the association of a 16S rRNA methyltransferase with a VEB enzyme, except for the identification of *rmtC* with bla_{VEB-6} (14).

This study investigated the occurrence of 16S rRNA methyltransferases in a *Salmonella enterica* serovar Thompson (H093960452) isolate and three *S. enterica* serovar Worthington isolates (H100680494, H100740257, and H095180621) selected from the Health Protection Agency Laboratory of Gastrointestinal Pathogens culture collection based on their ability to grow on Iso-Sensitest agar containing 500 mg/liter of amikacin (Table 1). PCR screening of the four isolates for the known methyltransferase genes identified *armA* in all of them. In addition, broth microdilution susceptibility testing revealed that the *S.* Worthington isolates exhibited high-level resistance to several cephalosporins and aztreonam (Table 1), suggesting the production of an ESBL. A series of multiplex PCRs were used to screen for the presence of genes encoding TEM-, SHV-, OXA-1/4/30/48-, CTX-M-1,3,9,8/25-, ACC-, FOX-, MOX-, DHA-, CIT-, EBC-, GES-, PER-, VEB-, IMP-, VIM-, and KPC-like β-lactamases and resulted in the identification of the bla_{VEB} and bla_{CMY} alleles (5). Primers for amplification of the entire genes were designed following alignment of the bla_{VEB} and bla_{CMY} nucleotide sequences deposited in GenBank and used to amplify and sequence the full coding sequences, which were subsequently cloned into the pCR-Blunt II TOPO vector (Invitrogen, Paisley, United Kingdom) and transformed into *Escherichia coli* TOP10 (Invitrogen). The nucleotide sequences were confirmed using vector-specific primers, and this revealed that the bla_{VEB} gene shared 99% sequence identity with bla_{VEB-5}, which was originally identified in *E. coli* in the United States and deposited in GenBank under accession number EF420108. The protein sequence was identical to that of previously identified VEB-5. The bla_{CMY} gene was identified as bla_{CMY-2} (accession number X91840). Pulsed-field gel electrophoresis (PFGE), performed according to the PulseNet Europe protocol (12), showed that the three *S.* Worthington isolates were identical (data not shown). Transfer of *armA* from the *S.* Thompson and *S.* Worthington isolates by conjugation was assessed using nalidixic acid-resistant *E. coli* K802N as the recipient and selecting with gentamicin at 50 mg/liter and nalidixic acid at 50 mg/liter. MICs of different antimicrobial agents were determined, by following the CLSI guidelines (4), for the wild-type strains, as well as for the transconjugants (Table 1), demonstrating that transconjugants BB1082 and BB1083 showed high-level resistance to aminoglycosides, cefotaxime, and aztreonam, respectively. PCRs confirmed the presence of *armA*, bla_{VEB-5}, and

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TABLE 1 MICs of selected antimicrobial agents for the strains used in this study

Strain	MIC (mg/liter)[a]										
	GEN	AMK	TOB	NEO	AMP	ATM	CTX	STR	TMP	STX	TET
S. Thompson H093960452	512	>1,024	>1,024	2	>1,024	≤0.5	≤0.5	32	>1,024	>1,024	512
S. Worthington											
H095180621	>1,024	>1,024	>1,024	128	>1,024	>1,024	256	16	≤0.5	>1,024	1
H100740257	>1,024	>1,024	>1,024	128	>1,024	>1,024	256	16	≤0.5	>1,024	1
H100680494	>1,024	>1,024	>1,024	64	>1,024	>1,024	256	16	≤0.5	>1,024	1
E. coli											
BB1082[b]	256	>1,024	512	4	>1,024	≤0.5	≤0.5	8	>1,024	>1,024	128
BB1083[b]	>1,024	>1,024	512	16	>1,024	>1,024	128	4	≤0.5	>1,024	1
K802N	≤0.5	≤0.5	≤0.5	1	4	≤0.5	≤0.5	4	≤0.5	2	1

[a] GEN, gentamicin; AMK, amikacin; TOB, tobramycin; NEO, neomycin; AMP, ampicillin; ATM, aztreonam; CTX, cefotaxime; STR, streptomycin; TMP, trimethoprim; STX, sulfamethoxazole; TET, tetracycline.
[b] Transconjugants BB1082 and BB1083 were obtained from *S.* Thompson H093960452 and *S.* Worthington H100680494, respectively.

Letter to the Editor

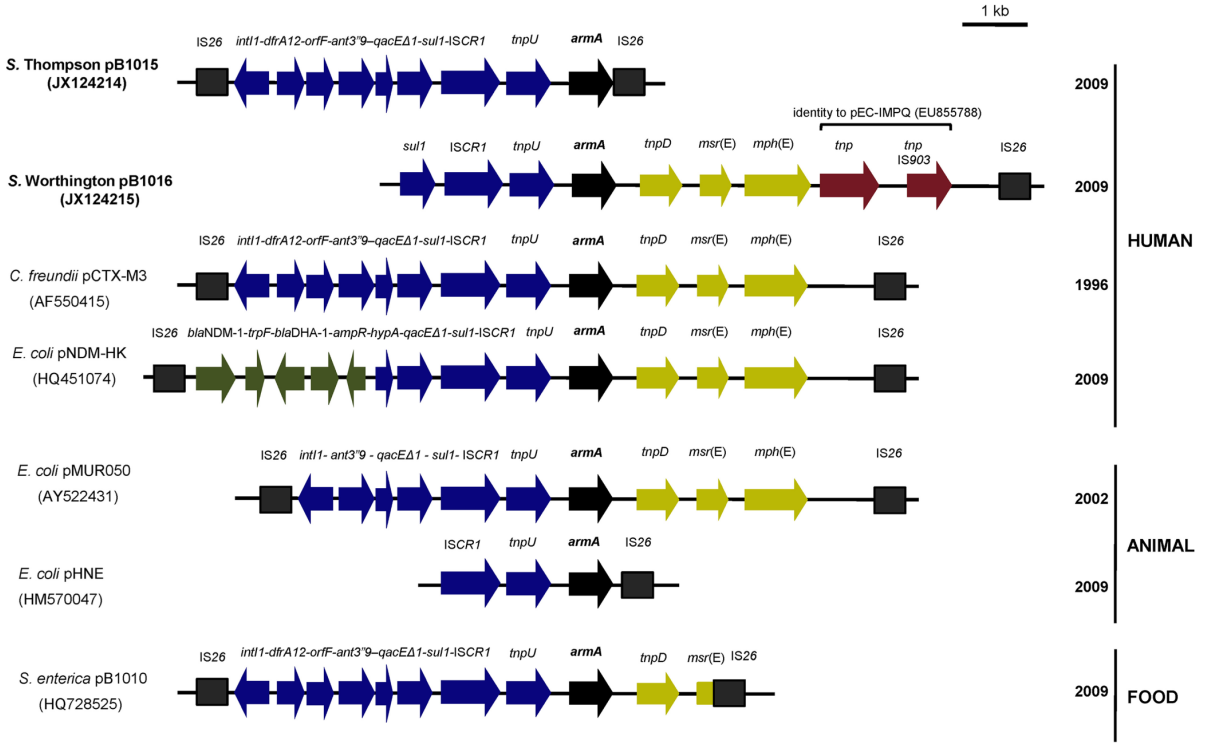

FIG 1 Scheme showing a Tn*1548*-like structure as the genetic environment of *armA* in pB1015 and pB1016 and comparison with the *armA* genetic environment previously described in human, animal, and food isolates. The year of isolation of each isolate is indicated. Arrows of the same color denote similarity within the same region between the different structures.

bla_{CMY-2} in BB1083, whereas only *armA* was amplified from BB1082. The latter also exhibited resistance to tetracycline (Table 1), and PCR and sequencing confirmed the presence of the *tetB* gene in this transconjugant. Plasmids from the wild-type strains and the transconjugants were analyzed by the S1-PFGE method (1) and replicon typing (3), showing that *armA* was located on pB1015, a 245-kb plasmid from the IncHI2 family in *S.* Thompson, whereas in *S.* Worthington an IncA/C plasmid of 170 kb named pB1016 bore *armA*, bla_{VEB-5}, and bla_{CMY-2}. In order to determine the genetic environment of the *armA* gene (8), PCR mapping for Tn*1548* was performed for *S.* Thompson and *S.* Worthington as previously described (10). A genetic structure related to Tn*1548* was found in both *S.* Thompson and *S.* Worthington to be the mobile element responsible for the spread of *armA* (Fig. 1).

Here we describe for the first time the association of ArmA with a VEB β-lactamase. This is also the first report of the bla_{VEB-5} gene in *Salmonella enterica*. These findings are of a great concern due to the combined presence of resistance to aminoglycosides and all β-lactams except carbapenems. Furthermore, these resistance determinants are located on the same plasmid, raising concern that further spread worldwide is possible. Further surveillance of these resistance genes in bacteria will help to slow down resistance to these clinically relevant antibiotics.

Nucleotide sequence accession number. The nucleotide sequence of the bla_{VEB-5} gene identified in this study has been deposited in GenBank under accession number JQ815440.

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Association of the novel aminoglycoside resistance determinant RmtF with NDM carbapenemase in Enterobacteriaceae isolated in India and the UK

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Objectives: 16S rRNA methyltransferases are an emerging mechanism conferring high-level resistance to clinically relevant aminoglycosides and have been associated with important mechanisms such as NDM-1. We sought genes encoding these enzymes in isolates highly resistant (MIC >200 mg/L) to gentamicin and amikacin from an Indian hospital and we additionally screened for the novel RmtF enzyme in 132 UK isolates containing NDM.

Methods: All highly aminoglycoside-resistant isolates were screened for *armA* and *rmtA-E* by PCR, with cloning experiments performed for isolates negative for these genes. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry was used to determine the methylation target of the novel RmtF methyltransferase. RmtF-bearing strains were characterized further, including susceptibility testing, PFGE, electroporation, PCR-based replicon typing and multilocus sequence typing of *rmtF*-bearing plasmids.

Results: High-level aminoglycoside resistance was detected in 140/1000 (14%) consecutive isolates of Enterobacteriaceae from India. ArmA, RmtB and RmtC were identified among 46%, 20% and 27% of these isolates, respectively. The novel *rmtF* gene was detected in 34 aminoglycoside-resistant isolates (overall prevalence 3.4%), most (59%) of which also possessed a bla_{NDM} gene; *rmtF* was detected in 6 NDM producers from the UK. It was found on different plasmid backbones. Four and two isolates showed resistance to tigecycline and colistin, respectively.

Conclusions: RmtF was often found in association with NDM in members of the Enterobacteriaceae and on diverse plasmids. It is of clinical concern that the RmtF- and NDM-positive strains identified here show additional resistance to tigecycline and colistin, current drugs of last resort for the treatment of serious bacterial infections.

Keywords: antimicrobial, resistance, 16S rRNA methyltransferases

Introduction

Aminoglycosides are considered to be critically important antibiotics by the World Health Organization.[1] Resistance is frequently mediated by aminoglycoside-modifying enzymes that are able to acetylate, phosphorylate or adenylate the antibiotic molecule.[2] Recently, methylation of the aminoacyl site of the 16S rRNA has been described as an acquired high-level resistance mechanism to clinically important aminoglycosides such as amikacin, tobramycin and gentamicin.[3] Since 2003, eight 16S rRNA methyltransferase genes—*armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD*, *rmtE*, *rmtF* and *npmA*—have been identified in several species of pathogenic bacteria worldwide,[4–6] being reported in most cases from human clinical isolates. The genes encoding these determinants are usually borne by mobile genetic elements and have been associated with mechanisms conferring resistance to other antibiotic classes, such as Qnr,[7] acquired AmpC[7] or extended-spectrum β-lactamases (ESBLs).[8–10] Recently

these methyltransferases have been found in association with the NDM-1 determinant that was first described for Swedish isolates obtained from a patient of Indian origin.[11–15] The recent appearance of multidrug-resistant bacteria with NDM-1 and other emerging resistance determinants such as the 16S rRNA methyltransferases is becoming an increasing clinical and public health threat.

This study was initially designed to determine the prevalence of 16S rRNA methyltransferases among clinical Enterobacteriaceae isolated in a hospital in India and their association with the emerging NDM-type carbapenemase. The novel 16S rRNA methyltransferase RmtF was evident in several Indian isolates and we therefore carried out an extensive screening for the *rmtF* gene in NDM-producing Enterobacteriaceae from the UK.

Methods

Bacterial isolates and antimicrobial susceptibility testing

A total of 1000 consecutive clinical Enterobacteriaceae strains recovered at the Sanjay Gandhi Postgraduate Institute of Medical Sciences in Lucknow, India, were analysed for their ability to grow on brain heart infusion (BHI) agar containing both 200 mg/L amikacin and 200 mg/L gentamicin. These strains were collected during 2010 (months of June, September, October and December) and January 2011. Clinical samples were urine, pus, sputum, CSF and blood. The Sanjay Gandhi Postgraduate Institute of Medial Sciences is an 800 bed tertiary care and referral hospital that draws patients from most of the states of northern India with the majority from the states of Uttar Pradesh and Bihar. All the isolates were recovered from patients admitted to the hospital in different medical and surgical disciplines. The MICs of ampicillin, amoxicillin/clavulanic acid, cefotaxime, cefotaxime/clavulanic acid, aztreonam, imipenem, imipenem/EDTA, meropenem, ciprofloxacin, tobramycin, amikacin, gentamicin, tigecycline and colistin were determined for aminoglycoside-resistant isolates by BSAC agar dilution methodology and interpreted according to BSAC version 10.2 criteria.

In addition, 132 NDM-producing Enterobacteriaceae received at the Antimicrobial Resistance and Healthcare Associated Infections (AMRHAI) Reference Unit, Health Protection Agency, UK, between 2009 and 2011 were screened for the presence of the newly discovered *rmtF* gene.

Table 1. Primers used in this study

Name	Sequence (5′→3′)	Amplicon size	Reference
armA.F	caaatggataagaatgatgtt	774 bp	3
armA.R	ttatttctgaaatccact		
rmtA.F	atgagctttgacgatgcccta	756 bp	16
rmtA.R	tcacttattccttttttatcatg		
rmtB.F	atgaacatcaacgatgccct	769 bp	8
rmtB.R	ccttctgattggcttatcca		
rmtC.F	cgaagaagtaacagccaaag	711 bp	17
rmtC.R	atcccaacatctctcccact		
rmtD.F	cggcacgcgattgggaagc	401 bp	17
rmtD.R	cggaaacgatgcgacgat		
rmtE.F	atgaatattgatgaaatggttgc	818 bp	18
rmtE.R	tgattgatttcctccgtttttg		
rmtF.F	gcgatacagaaaaccgaagg	589 bp	this work
rmtF.R	accagtcggcatagtgcttt		
NDM.F	tctcgacatgccgggtttcgg	475 bp	this work
NDM.R	accgagattgccgagcgactt		
CTX-Muni-F	cgatgtgcagtaccagtaa	585 bp	19
CTX-Muni-R	ttagtgaccagaatcagcgg		
rmtFUPS.F	gggcgtacacaattcattca	1112 bp	this work
rmtFUPS.R	catacgtcccgcagattctt		
rmtFDWNS.F	cgcttctgatgaagctcctg	1481 bp	this work
rmtFDWNS.R	tggtgatgtcgatggaaaag		

PCR and strain typing

PCRs for genes encoding the 16S methyltransferases (*armA* and *rmtA-F*), CTX-M ESBLs and NDM-type carbapenemase were carried out using primers listed in Table 1 and lysed cells as DNA template. A multiplex PCR was performed for the detection of acquired AmpC genes as previously described.[20] Sequences of the designed primers for the study of the *rmtF* genetic environment can be found in Table 1. The phylogenetic groups of *Escherichia coli* isolates were determined as previously described.[21]

Relatedness among the isolates was assessed by PFGE. Genomic DNA was embedded in agarose blocks and digested with XbaI (Roche Diagnostics, Mannheim, Germany). PFGE was carried out on a CHEF-DR III device (Bio-Rad, Hercules, CA, USA) for 30 h at 6 V/cm^2 and 12°C with an initial pulse time of 5 s and a final pulse time of 35 s. A dendrogram of strain relatedness was constructed with BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium) for the *Klebsiella pneumoniae* isolates.

DNA analysis and manipulation

Cloning experiments were carried out by digesting plasmid DNA with EcoRI restriction enzyme and ligating the resulting fragments into pUC19 vector. The ligation mixture was transformed into DH5α cells, which were then plated on agar containing ampicillin (50 mg/L) and gentamicin (10 mg/L). A CloneJET PCR cloning kit (Fermentas International Inc.) was used to clone *rmtF* along with its promoter region, previously determined with the Softberry online tool.

Plasmid characterization

Plasmid DNA was extracted from clinical isolates using a Plasmid Midi kit (Qiagen Inc., Chatsworth, CA, USA). Transformation experiments were conducted with Alpha-Select Electrocompetent cells (Bioline, London, UK) following the manufacturer's instructions. Plasmids were classified according to their incompatibility group using a PCR-based replicon typing kit (Diatheva, Fano, Italy). Plasmid multilocus sequence typing (pMLST) was used to further characterize IncN plasmids.[22] In order to determine plasmid size, agarose blocks containing total DNA were digested with S1 nuclease (Promega, Madison, WI, USA) following the manufacturer's instructions. Blocks were then loaded into a 1% agarose gel and PFGE was performed as previously described.[16]

Analysis of bacterial rRNA by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry

Total rRNA was extracted from ribosomal particles isolated from *rmtF*-bearing transformant *E. coli* BB1284.[23] The 16S rRNA sequence from C1378–G1432 was protected by hybridization to a complementary deoxyoligonucleotide and the unhybridized RNA was digested with mung bean nuclease (NE Biolabs, Ipswich, MA, USA) and RNase A (Sigma-Aldrich, St Louis, MO, USA) as previously described.[24] The rRNA fragment was isolated by gel electrophoresis[24,25] and digested overnight

at 37°C with 3 units of RNase T1 (Roche Diagnostics, Mannheim, Germany) in 2 μL of 50 mM 3-hydroxypicolinic acid. For analysing the fragments, MALDI mass spectrometry (Voyager Elite, PerSeptive Biosystems) was performed, recording in reflector and positive ion mode.[26] The program m/z (Proteometrics Ltd, New York, NY, USA) was used for interpreting the spectra.

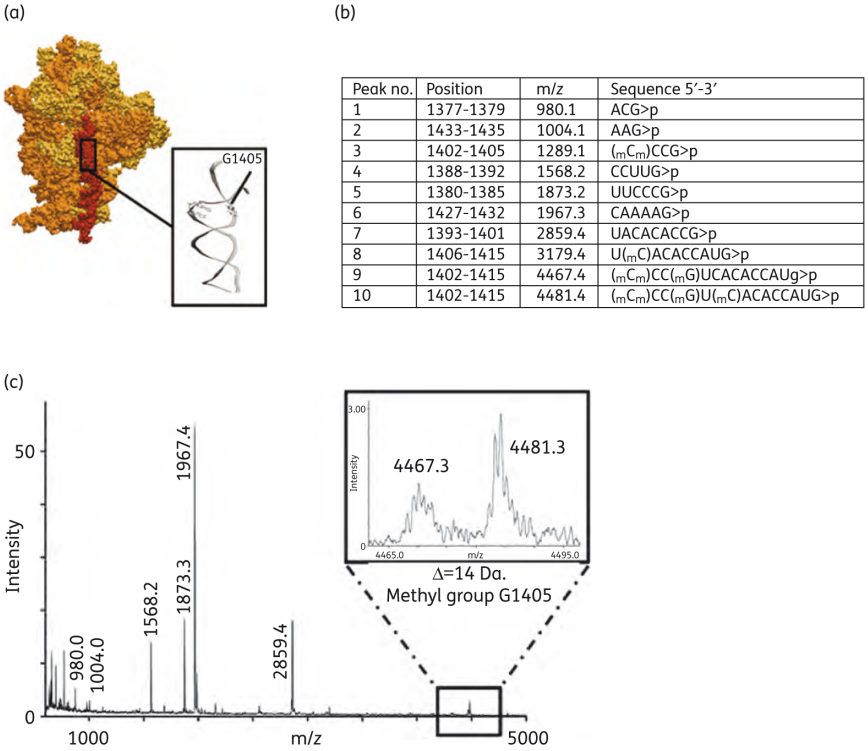
Results

16S rRNA methyltransferases are highly prevalent among clinical isolates in India

One-hundred-and-forty (14%) of 1000 consecutive clinical isolates of Enterobacteriaceae from the Sanjay Gandhi Postgraduate Institute of Medical Sciences were highly resistant to gentamicin (MIC >200 mg/L) and amikacin (MIC >200 mg/L). The isolates were recovered from urine, pus, sputum, CSF or blood. The methyltransferases ArmA, RmtB and RmtC were detected in 64 (46%), 28 (20%) and 37 (27%) of the 140 isolates, respectively, including *E. coli*, *K. pneumoniae*, *Citrobacter freundii*, *Citrobacter koseri*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Proteus mirabilis* and *Providencia* spp. More than one methyltransferase gene was detected in a few isolates: two isolates harboured ArmA and RmtB, three isolates harboured ArmA and RmtC and five isolates harboured RmtB and RmtC. The NDM-type carbapenemase was identified in 90 (64%) isolates.

Novel RmtF confers high-level resistance to aminoglycosides through methylation of nucleotide G1405 in the 16S rRNA

Nineteen of the 140 isolates highly resistant to aminoglycosides were negative by PCR for *armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD* and *rmtE*. However, high-level aminoglycoside resistance was transferred to an *E. coli* recipient by electroporation using plasmid DNA extracted from two representatives of these isolates (BB1088 and BB1265). Cloning and subsequent sequencing identified the novel aminoglycoside resistance methylase gene, *rmtF*, on a DNA fragment of ~4.6 kb, which was sequenced. Its immediate genetic environment consisted of an *insE* transposase downstream of *rmtF* with an inverted repeat duplicated immediately upstream of this new determinant. RmtF is a recently described protein of 260 amino acids that shares the highest amino acid identity (46%) with RmtD and it is able to confer high-level resistance (MIC



Peak no.	Position	m/z	Sequence 5′-3′
1	1377-1379	980.1	ACG>p
2	1433-1435	1004.1	AAG>p
3	1402-1405	1289.1	($_m$C$_m$)CCG>p
4	1388-1392	1568.2	CCUUG>p
5	1380-1385	1873.2	UUCCCG>p
6	1427-1432	1967.3	CAAAAG>p
7	1393-1401	2859.4	UACACACCG>p
8	1406-1415	3179.4	U($_m$C)ACACCAUG>p
9	1402-1415	4467.4	($_m$C$_m$)CC($_m$G)UCACACCAUg>p
10	1402-1415	4481.4	($_m$C$_m$)CC($_m$G)U($_m$C)ACACCAUG>p

Figure 1. (a) Three-dimensional structure of the 30S ribosomal subunit.[34] Yellow and orange represent proteins and 16S rRNA, respectively. Helix 44 is shown in red and the enlarged view shows the aminoglycoside binding pocket. (b) The theoretical monoisotopic masses of RNase T1 digestion products of trinucleotides and larger from the *E. coli* 16S rRNA sequence C1378–G1432. (c) MALDI mass spectrometry spectrum of this region from *E. coli* expressing *rmtF*, showing the empirically measured masses. Methylation at G1405 prevents RNase T1 cleavage at this nucleotide and gives rise to the longer fragments at *m/z* 4467 and 4481. The *m/z* 4467 fragment lacks the intrinsic methylation at C1407,[35] which could be partially obstructed by the action of RmtF.

Table 2. Features of the clinical isolates positive for the *rmtF* methyltransferase gene

Species	Origin	NDM type	Other resistance genes[b]	Plasmid bearing *rmtF*
K. pneumoniae BB1088	India	+	—	untypeable
K. pneumoniae BB1255	India	+	*armA*, bla_{CTX-M}	untypeable
K. pneumoniae BB1257	India	−	bla_{CTX-M}	IncN ST14
K. pneumoniae BB1258	India	+	*armA*, bla_{CTX-M}	untypeable
K. pneumoniae BB1259	India	−	bla_{CTX-M}	IncR
K. pneumoniae BB1260	India	−	bla_{CTX-M}	untypeable
K. pneumoniae BB1261	India	−	—	IncR
K. pneumoniae BB1089	India	+	*rmtB*, bla_{CTX-M}, bla_{CIT}	non-transformable
K. pneumoniae BB1262	India	+	bla_{CTX-M}	untypeable
K. pneumoniae BB1263	India	+	bla_{CTX-M}	non-transformable
K. pneumoniae BB1090	India	+	bla_{CTX-M}	IncR
K. pneumoniae BB1269	India	−	bla_{CTX-M}	untypeable
K. pneumoniae BB1270	India	+	bla_{CTX-M}	untypeable
K. pneumoniae BB1274	India	+	*rmtC*, bla_{CTX-M}	untypeable
K. pneumoniae BB1275	India	+	*armA*, bla_{CTX-M}	untypeable
K. pneumoniae BB1282	India	+	*armA*, bla_{CTX-M}, bla_{DHA}	IncN ST14
K. pneumoniae BB1283	India	+	*armA* bla_{CTX-M}, bla_{DHA}	IncN ST14
K. pneumoniae H113520208	UK	+	bla_{CTX-M}	untypeable
K. pneumoniae H100500310	UK	+	bla_{CTX-M}	untypeable
K. pneumoniae H110360516	UK	+	bla_{CTX-M}	untypeable
K. pneumoniae H113840625	UK	+	bla_{CTX-M}	untypeable
K. pneumoniae H100500328	UK	+	bla_{CTX-M}	untypeable
K. pneumoniae H090480398	UK	+	*rmtC*, bla_{CTX-M}, bla_{CIT}	untypeable
E. coli B2[a] BB1265	India	−	bla_{CTX-M}	IncN ST14
E. coli B2 BB1266	India	+	*rmtC*, bla_{CTX-M}, bla_{CIT}	IncN ST14
E. coli B2 BB1268	India	−	bla_{CTX-M}	non-transformable
E. coli B2 BB1272	India	−	bla_{CTX-M}	IncN ST14
E. coli B1 BB1276	India	+	*rmtC*, bla_{CTX-M}, bla_{CIT}	non-transformable
E. coli B2 BB1277	India	−	bla_{CTX-M}	IncN ST14
E. coli B2 BB1278	India	+	*rmtB*, bla_{CTX-M}	IncN ST14
E. coli B1 BB1093	India	+	*armA*, bla_{CTX-M}, bla_{CIT}, bla_{DHA}	non-transformable
E. coli A BB1279	India	−	*armA*, bla_{CTX-M}	non-transformable
E. coli B1 BB1280	India	+	*armA*, bla_{CTX-M}	untypeable
C. freundii BB1256	India	−	*armA*, bla_{CTX-M}	non-transformable
C. freundii BB1264	India	−	bla_{CTX-M}	IncN ST14
C. freundii BB1281	India	+	*armA*, bla_{CTX-M}	untypeable
E. cloacae BB1267	India	+	bla_{CTX-M}	untypeable
E. cloacae BB1092	India	+	bla_{CTX-M}	non-transformable
E. cloacae BB1271	India	+	bla_{CTX-M}	non-transformable
E. cloacae BB1273	India	−	—	untypeable

[a]*E. coli* phylogenetic group.
[b]ESBL, acquired AmpC and 16S rRNA methyltransferase genes.

>200 mg/L) to the 4,6-disubstituted 2-deoxystreptamine aminoglycosides, such as gentamicin, amikacin and tobramycin.[5,6] Using MALDI mass spectrometry we found that RmtF confers high-level aminoglycoside resistance by adding a methyl group at position G1405 of the bacterial 16S rRNA (Figure 1).

rmtF detected in diverse Enterobacteriaceae in India and the UK

The 140 Indian isolates were screened for the *rmtF* gene using PCR (see Table 1 for *rmtF*-specific primer sequences). A total of 34 isolates were found to carry *rmtF*; these comprised 17 *K. pneumoniae*, 10 *E. coli* (six of them belonging to the phylogenetic group B2 related with virulent extra-intestinal strains), 3 *C. freundii* and 4 *E. cloacae* isolates (Table 2). The 17 *K. pneumoniae* isolates had 15 different PFGE profiles using a cut-off at 85% genetic similarity. Two pairs of isolates, collected consecutively, shared an identical profile. The first pair was isolated from sputum and pus and the second pair was isolated from pus and urine; each isolate was from a different patient and thus represents possible cross-infection. Following the Tenover *et al.*[27] PFGE interpretation, four *E. coli* isolates were

Table 3. MICs for the 40 clinical isolates bearing *rmtF* in this study

	MIC (mg/L)													
Strain no.	AMP	AMC	CTX	CTX/CLA	ATM	IPM	IPM+E	MEM	CIP	TOB	AMK	GEN	TGC	CST
BB1088	>64	64	>256	>32	>64	32	0.25	32	>8	>32	>64	>32	0.5	1
BB1255	>64	64	>256	>32	>64	32	1	32	>8	>32	>64	>32	1	1
BB1256	>64	64	>256	>32	>64	1	1	1	>8	>32	>64	>32	0.5	1
BB1257	>64	64	>256	8	>64	1	0.25	0.125	>8	>32	>64	>32	2	1
BB1258	>64	64	>256	>32	>64	32	0.5	32	>8	>32	>64	>32	1	1
BB1259	>64	32	>256	>32	>64	2	0.25	2	>8	>32	>64	>32	2	1
BB1260	>64	32	>256	1	>64	1	0.5	0.5	>8	>32	>64	>32	0.5	1
BB1261	>64	32	>256	0.5	>64	0.5	0.5	0.25	>8	>32	>64	>32	1	1
BB1089	>64	>64	>256	>32	>64	64	4	>32	>8	>32	>64	>32	2	≤0.5
BB1262	>64	64	>256	>32	>64	32	1	>32	>8	>32	>64	>32	2	1
BB1263	>64	64	>256	>32	>64	64	1	32	>8	>32	>64	>32	2	1
BB1264	>64	64	>256	0.25	>64	2	1	≤0.06	>8	>32	>64	>32	0.5	≤0.5
BB1265	>64	32	>256	0.25	>64	1	1	≤0.06	>8	>32	>64	>32	0.5	1
BB1266	>64	64	>256	>32	>64	32	0.5	>32	>8	>32	>64	>32	≤0.25	1
BB1267	>64	64	>256	16	64	32	1	32	>8	>32	>64	>32	0.5	1
BB1090	>64	>64	>256	>32	>64	128	4	>32	>8	>32	>64	>32	2	≤0.5
BB1268	>64	64	>256	2	>64	1	0.5	0.125	>8	>32	>64	>32	0.5	≤0.5
BB1269	>64	32	>256	0.25	>64	0.25	0.5	≤0.06	>8	>32	>64	>32	0.5	1
BB1270	>64	64	>256	>32	>64	32	0.5	32	>8	>32	>64	>32	2	4
BB1092	>64	>64	>256	>32	>64	16	2	32	>8	>32	>64	>32	2	16
BB1271	>64	>64	>256	>32	>64	32	2	>32	>8	>32	>64	>32	2	1
BB1272	>64	>64	>256	>32	>64	1	1	32	>8	>32	>64	>32	2	1
BB1273	>64	64	>256	>32	>64	1	1	0.125	>8	>32	>64	>32	0.5	≤0.5
BB1274	>64	64	>256	>32	>64	32	1	>32	>8	>32	>64	>32	1	1
BB1275	>64	64	>256	>32	>64	32	0.25	32	>8	>32	>64	>32	1	1
BB1276	>64	64	>256	>32	>64	32	0.25	>32	>8	>32	>64	>32	≤0.25	≤0.5
BB1277	>64	32	>256	0.5	>64	0.5	1	≤0.06	>8	>32	>64	>32	0.5	≤0.5
BB1278	>64	64	>256	1	>64	1	0.5	≤0.06	>8	>32	>64	>32	0.5	1
BB1093	>64	64	>256	>32	>64	32	0.5	32	>8	>32	>64	>32	0.5	1
BB1279	>64	32	>256	>32	>64	0.5	0.25	1	>8	>32	>64	>32	2	1
BB1280	>64	64	>256	>32	>64	64	0.25	>32	>8	>32	>64	>32	0.5	1
BB1281	>64	64	>256	>32	>64	64	0.5	32	>8	>32	>64	>32	1	1
BB1282	>64	>64	>256	>32	>64	64	4	>32	>8	>32	>64	>32	4	1
BB1283	>64	>64	>256	>32	>64	64	4	>32	>8	>32	>64	>32	4	1
H113520208	>64	64	>256	>32	>64	64	0.25	>32	>8	>32	>64	>32	2	≤0.5
H100500310	>64	64	>256	>32	>64	128	0.25	>32	>8	>32	>64	>32	1	≤0.5
H110360516	>64	64	>256	>32	>64	32	0.5	32	>8	>32	>64	>32	4	≤0.5
H113840625	>64	64	>256	>32	>64	16	0.5	32	>8	>32	>64	>32	2	≤0.5
H100500328	>64	64	>256	>32	>64	64	0.06	>32	>8	>32	>64	>32	16	1
H090480398	>64	64	>256	>32	>64	64	0.25	>32	>8	>32	>64	>32	2	≤0.5

AMP, ampicillin; AMC, amoxicillin/clavulanic acid; CTX, cefotaxime; CTX/CLA, cefotaxime/clavulanic acid; ATM, aztreonam; IPM, imipenem; IPM+E, imipenem+EDTA; MEM, meropenem; CIP, ciprofloxacin; TOB, tobramycin; AMK, amikacin; GEN, gentamicin; TGC, tigecycline; CST, colistin.

indistinguishable (no band differences), and compared with them the six other *E. coli* were unrelated (seven or fewer fragment differences). Of these six, two were identical, making a total of six different profiles. Two *E. cloacae* out of four shared identical PFGE patterns, while all the *C. freundii* isolates were unrelated.

More NDM-positive Enterobacteriaceae have been detected in the UK than any other European country,[28] and because 20 of 34 *rmtF*-positive Indian isolates also harboured bla_{NDM}, we screened 132 NDM-producing isolates from the UK for *rmtF* and other 16S rRNA methyltransferases. The *armA*, *rmtB* or *rmtC* genes were detected in 44 (33%), 1 (0.8%) and 47 (36%) isolates, respectively. Eighteen (14%) isolates harboured both *armA* and *rmtC*, while 1 (0.8%) harboured *armA* and *rmtB*. Six (5%) UK isolates, all *K. pneumoniae* harboured *rmtF* (Table 2). These had been isolated from blood, urine or rectal swabs from patients in different

hospitals across the UK. PFGE showed that these UK isolates were distinct from each other (≤85% of genetic similarity) except for three of them sharing 88% genetic similarity. Most of these isolates were unrelated to the *rmtF*-positive *K. pneumoniae* isolates from India; however, one UK isolate (H113840625) was closely related (88% genetic similarity) with an Indian isolate (BB1269) (data not shown).

Analysis of plasmids encoding RmtF

The *rmtF* gene was transferred by electroporation to an *E. coli* recipient from 31 of 40 isolates; transformants could not be obtained for 9 Indian isolates. Plasmid *rep* typing revealed that most *rmtF*-bearing plasmids (in 19 isolated strains, from India and the UK) were untypeable. However, *rmtF* was located on IncN plasmids in five *E. coli*, three *K. pneumoniae* and one *C. freundii*, all from India, and on IncR plasmids in three *K. pneumoniae* from India (Table 2). All of the IncN plasmids were analysed by pMLST and belonged to a new sequence type, ST14.

To characterize the genetic environment of *rmtF*, two PCR assays were developed (Table 1) to amplify regions of ~1.2 and 1.5 kb upstream and downstream of *rmtF*, respectively. Twenty-one (52.5%) isolates of different species and bearing *rmtF* on distinct plasmid backbones were positive for both PCR products and 16 (40%) were positive only for the upstream fragment. Three isolates were negative in both PCR assays.

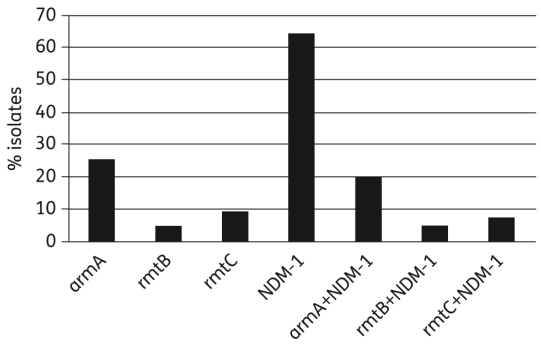


Figure 2. Distribution of NDM and different 16S rRNA methyltransferases among the clinical isolates bearing *rmtF* from India and the UK.

RmtF is associated with emerging resistance mechanisms, including NDM carbapenemase

Antibiotic susceptibility profiles for the 34 Indian and 6 UK RmtF-positive isolates revealed phenotypes compatible with other co-resident resistance determinants (Table 3). Furthermore, PCR screening showed that in several cases RmtF was co-resident in the same isolate with a second 16S rRNA methyltransferase—ArmA, RmtB or RmtC (Figure 2).

Twenty of the 34 Indian RmtF isolates (59%) were NDM positive; these were not selected on the basis of carbapenem resistance. The six UK RmtF isolates were detected during targeted screening of NDM-positive isolates from 2009 (one isolate), 2010 (one isolate) and 2011 (four isolates). As shown by the S1-PFGE technique performed with the transformants, the NDM gene and *rmtF* were genetically linked on a non-typeable plasmid of ~120 kb in six strains from India (four *K. pneumoniae*, one *E. cloacae* and one *C. freundii*) and in three of the six *K. pneumoniae* from the UK (data not shown). In addition, NDM was found co-resident with two methyltransferases (RmtF plus ArmA, RmtB or RmtC) in various isolates (Figure 3).

A $bla_{CTX\text{-}M}$ gene was detected in 37 of 40 (93%) isolates, while an acquired AmpC gene was detected in 7 of 40 (17.5%) isolates.

Four *rmtF*-bearing *K. pneumoniae* were resistant to tigecycline (MICs 4–16 mg/L) and 14 isolates (10 *K. pneumoniae*, 2 *E. cloacae* and 2 *E. coli*) had intermediate resistance (2 mg/L)

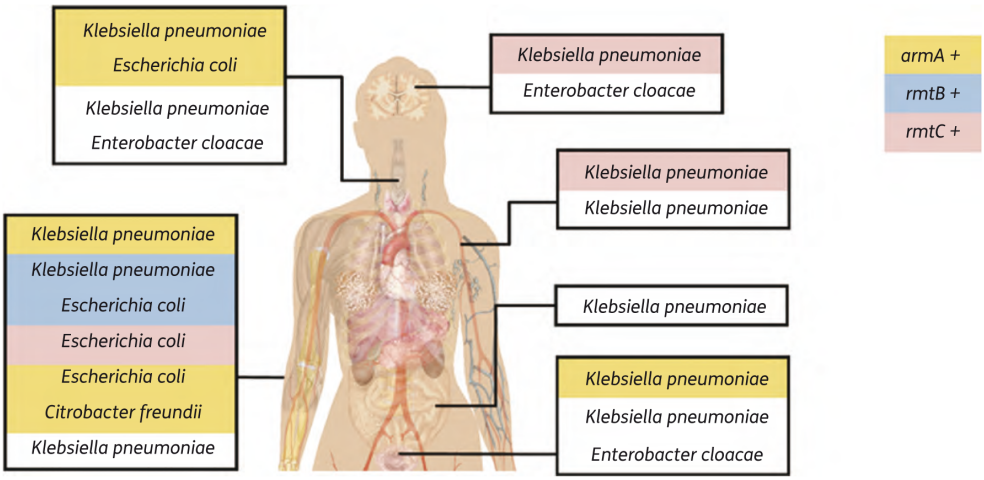


Figure 3. Source of *rmtF*-positive species isolated in this study bearing NDM (pus, sputum, CSF, blood, rectal swab and urine). Colours indicate concomitant presence of a second 16S rRNA methyltransferase gene. [Female shadow with organs: image made available by Mikael Häggström (2011) via Wikimedia Commons under a CC0 licence].

(Table 2). Colistin resistance was detected in one *K. pneumoniae* (4 mg/L) and one *E. cloacae* (16 mg/L), both isolated in India. The *E. cloacae* (BB1092) was recovered from sputum and was an NDM producer; its PFGE profile was identical to that of a colistin-susceptible (1 mg/L) and NDM-producing isolate (BB1271) from CSF of a different patient.

Discussion

The 16S rRNA methyltransferases pose a public health threat because they confer broad and high-level resistance to most clinically available aminoglycosides. Some of the 16S rRNA methyltransferases presumably originated as intrinsic, chromosomally encoded enzymes in environmental *Actinomycetes*, serving to protect these bacteria from the aminoglycosides that they produce.[29] In recent years, however, diverse 16S rRNA methyltransferases have emerged as acquired resistance determinants in several genera of the Enterobacteriaceae and have been detected not only in humans, but also in pets,[30] livestock[31] and food.[16,32]

A new 16S methyltransferase, RmtF, was recently identified in a single *K. pneumoniae* isolate from La Réunion Island.[5] In this study we found that 14% of consecutive Enterobacteriaceae from an Indian hospital were highly resistant to aminoglycosides and identified many with 16S methyltransferases, of which 34 strains carried RmtF. Many (59%) of these RmtF-positive Indian isolates also contained an NDM carbapenemase, so we screened NDM-positive Enterobacteriaceae from the UK to explore this association further, finding six more RmtF producers.

Galimand *et al.*[5] deduced that RmtF is a G1405 methyltransferase based on amino acid identity with other G1405 methyltransferases. However, we have demonstrated in this study the mechanism of action of this novel determinant at the molecular level. The overall low level of amino acid identity shared between the 16S rRNA methyltransferases identified in clinical isolates of Gram-negative bacteria and the *Actinomycetes* suggests further novel 16S rRNA methyltransferases may be identified in clinical isolates in the future.

We identified RmtF in multiple strains of four different bacterial genera located on at least three different plasmid backbones. This diversity suggests that, like NDM carbapenemase, this novel aminoglycoside resistance determinant escaped unrecognized into Enterobacteriaceae some time ago.

Multidrug-resistant organisms, including those expressing resistance to carbapenems, are becoming increasingly prevalent worldwide. The identification of RmtF co-resident in strains harbouring ESBLs, acquired AmpC enzymes, the NDM carbapenemase and fluoroquinolone-resistance mechanisms not only leads to the potential for co-selection and maintenance of resistance by the use of other antibiotics, but also seriously compromises the treatment of life-threatening infections caused by Gram-negative organisms. The majority of RmtF-producing isolates remain susceptible to tigecycline and colistin, as do most strains harbouring NDM enzymes.[14,28] However, we observed clinical resistance to both tigecycline and colistin and intermediate resistance (2 mg/L) to tigecycline among isolates harbouring both RmtF and NDM, as has been previously noted in NDM producers.[28,33]

In conclusion, we identified here the emergence of a novel antimicrobial resistance determinant, RmtF, associated with NDM carbapenemase in Enterobacteriaceae isolated in India and the UK. Collaborative multinational programmes are crucial if further development of antimicrobial resistance is to be delayed.

The nucleotide sequence for the DNA fragment with *rmtF* cloned in this study has been deposited in GenBank under accession number JQ955744.

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Transparency declarations

None to declare.

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4) *Klebsiella pneumoniae* Sequence Type 11 from companion animals bearing ArmA methyltransferase, DHA-1 β-lactamase and QnrB4

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Klebsiella pneumoniae Sequence Type 11 from Companion Animals Bearing ArmA Methyltransferase, DHA-1 β-Lactamase, and QnrB4

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Seven *Klebsiella pneumoniae* isolates from dogs and cats in Spain were found to be highly resistant to aminoglycosides, and ArmA methyltransferase was responsible for this phenotype. All isolates were typed by multilocus sequence typing (MLST) as ST11, a human epidemic clone reported worldwide and associated with, among others, OXA-48 and NDM carbapenemases. In the seven strains, *armA* was borne by an IncR plasmid, pB1025, of 50 kb. The isolates were found to coproduce DHA-1 and SHV-11 β-lactamases, as well as the QnrB4 resistance determinant. This first report of the ArmA methyltransferase in pets illustrates their importance as a reservoir for human multidrug-resistant *K. pneumoniae*.

Aminoglycosides are widely used for the treatment of various bacterial infections due to Gram-positive and Gram-negative bacteria. Resistance to these antibiotics is frequently mediated by modifying enzymes that are able to acetylate, phosphorylate or adenylate the antibiotic molecule (1). Recently, 16S rRNA methyltransferases (ArmA, RmtA to -F, and NmpA) have been described as a new high-level aminoglycoside resistance mechanism among Gram-negative pathogenic bacteria (2, 3, 4). Since 2003, these methyltransferases have been reported worldwide, usually from human clinical isolates, except for ArmA and RmtB, reported from chickens (5) and pigs (6, 7, 8), and ArmA and RmtC, reported from food isolates (9, 10). There was no report of a 16S rRNA methyltransferase in bacteria isolated from house pets until 2011, when RmtB was found in several *Enterobacteriaceae* collected from pets in China (11).

The aim of this study was to find the genetic determinant responsible for the high-level resistance to clinically important aminoglycosides, such as gentamicin and amikacin, in seven *Klebsiella pneumoniae* strains isolated from pets (dogs and cats) at the Faculty of Veterinary Medicine in Madrid, Spain. Strains were collected throughout 2008, 2009, and 2010 from different animals with diverse diseases, all referred from the same veterinary surgery (Table 1). Pulsed-field gel electrophoresis (PFGE) typing was performed with all isolates (9) and showed a high genetic relatedness between them. *K. pneumoniae* multilocus sequence typing (MLST) was performed by using the primers described by Diancourt et al. (12) except for a different rpoB-forward primer (5′-TCTGACCCGTGAGCGCGCAGGCT). Allelic profiles and sequence types (STs) were verified at http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html. A unique allelic profile was obtained with the seven isolates, corresponding to ST11. ST11 is an epidemic clone of *K. pneumoniae* that has been isolated from humans worldwide, and it is associated with the spread of resistance determinants such as OXA-48 or NDM (13, 14, 15). The MICs, determined and interpreted according to Clinical and Laboratory Standards Institute guidelines (16, 17), showed high-level resistance to all 4,6-disubstituted 2-deoxystreptamine aminoglycosides, as well as to ampicillin, ceftazidime, sulfamethoxazole, tetracycline, chloramphenicol, and fluoroquinolones (Table 2).

Screening of 16S rRNA methyltransferase-encoding genes was performed by PCR and sequencing (9), revealing the presence of the *armA* gene in the seven isolates. Transfer of this gene into a laboratory *Escherichia coli* INVF′ strain was carried out by transformation using plasmid DNA extraction (Plasmid Midi Kit; Qiagen, Inc., Chatworth, CA) and demonstrated that *armA* was borne by a plasmid in all the *K. pneumoniae* strains. In order to elucidate β-lactam and quinolone resistance determinants, multiplex PCR assays for TEM, SHV, CMY, DHA, and Qnr genes were performed with wild-type and transformant bacteria (18, 19). bla_{SHV-11} was identified in the seven *K. pneumoniae* isolates, although it was absent in the corresponding transformants. A bla_{DHA} gene was amplified in the seven wild-type isolates and their transformants, and it was confirmed by sequencing to be bla_{DHA-1}. The bla_{DHA-1} gene was coharbored by the same plasmid as *armA* in the seven *K. pneumoniae* isolates. A *qnrB4* gene was detected in the wild-type strains, and it was found with *armA* and bla_{DHA-1} in the same plasmid in five strains (BB1097, BB1099, BB1101, BB1102, and BB1103) (Table

TABLE 1 Features of the seven *K. pneumoniae* isolates investigated in this study

Isolate no.	Source	Date of isolation	Sample	Plasmid bearing *armA*	Other resistance gene(s) in plasmid
BB1097	Cat	2009	Urine	pB1025	bla_{DHA-1}, *qnrB4*
BB1098	Dog	2009	Urine	pB1025-1	bla_{DHA-1}
BB1099	Dog	2009	Urine	pB1025	bla_{DHA-1}, *qnrB4*
BB1100	Dog	2008	Urine	pB1025-1	bla_{DHA-1}
BB1101	Cat	2009	Urine	pB1025	bla_{DHA-1}, *qnrB4*
BB1102	Dog	2009	Urine	pB1025	bla_{DHA-1}, *qnrB4*
BB1103	Dog	2010	Abscess	pB1025	bla_{DHA-1}, *qnrB4*

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TABLE 2 MICs for *K. pneumoniae* isolates and transformants

Strain[a]	MIC (mg/liter)[b]														
	AMK	AMP	CAZ	CHL	CIP	CST	CTX	FFN	GEN	KAN	NAL	SXT	STR	TET	TMP
K. pneumoniae BB1097	>512	>32	16	>64	>8	≤2	>4	16	512	>128	>64	>1024	32	>64	>32
E. coli BB1104	>512	16	2	>64	0.015	≤2	2	≤2	512	>128	≤4	≤8	>128	64	≤0.5
K. pneumoniae BB1098	>512	>32	4	>64	>8	≤2	1	16	512	>128	>64	>1024	32	>64	>32
E. coli BB1105	>512	16	2	≤2	≤0.008	≤2	≤0.5	≤2	512	>128	≤4	≤8	>128	32	≤0.5
K. pneumoniae BB1099	>512	>32	16	>64	>8	≤2	>4	16	>512	>128	>64	>1,024	16	2	>32
E. coli BB1106	>512	16	2	≤2	0.015	≤2	≤0.5	≤2	512	>128	≤4	≤8	>128	≤1	≤0.5
K. pneumoniae BB1100	>512	>32	8	>64	>8	≤2	2	16	>512	>128	>64	>1,024	32	2	>32
E. coli BB1107	>512	16	2	≤2	≤0.008	≤2	≤0.5	≤2	512	>128	≤4	≤8	>128	≤1	≤0.5
K. pneumoniae BB1101	>512	>32	4	>64	>8	≤2	1	16	>512	>128	>64	>1024	32	>64	>32
E. coli BB1108	>512	32	2	≤2	0.015	≤2	≤0.5	≤2	>512	>128	≤4	≤8	>128	64	≤0.5
K. pneumoniae BB1102	>512	>32	8	>64	>8	≤2	>4	8	>512	>128	>64	>,1024	32	>64	>32
E. coli BB1109	>512	16	1	≤2	0.015	≤2	≤0.5	≤2	512	>128	≤4	≤8	>128	64	≤0.5
K. pneumoniae BB1103	>512	>32	8	>64	>8	≤2	2	16	512	>128	>64	>1,024	32	>64	>32
E. coli BB1110	>512	16	2	≤2	0.015	≤2	≤0.5	≤2	512	>128	≤4	≤8	>128	64	≤0.5
E. coli INVF'	1	2	≤0.25	≤2	≤0.008	≤2	≤0.5	≤2	≤0.5	≤4	≤4	≤8	>128	≤1	≤0.5

[a] The rows show each *K. pneumoniae* strain isolated in this study followed by its respective transformant bearing a plasmid with *armA*.
[b] AMK, amikacin; AMP, ampicillin; CAZ, ceftazidime; CHL, chloramphenicol; CIP, ciprofloxacin; CST, colistin; CTX, cefotaxime; FFN, florfenicol; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; SXT, trimethoprim-sulfamethoxazole; STR, Streptomycin; TET, tetracycline; TMP, trimethoprim.

1). Interestingly, a report of a multidrug resistance plasmid from *K. pneumoniae* in China carrying a 25-kb region with *armA*, bla_{DHA-1}, and *qnrB4*, pKP048, was recently published (GenBank accession number FJ628167) (20), and an identical region was subsequently detected in several *K. pneumoniae* plasmids from Taiwan (21). With the aim of ascertaining whether our isolates bore the pKP048 plasmid or a derivative thereof, three PCR products of ca. 3.2, 1.1, and 1.2 kb were developed by designing 3 pairs of primers along the genetic environment of *armA*, bla_{DHA-1}, and *qnrB4* in pKP048. The five strains with *armA*, bla_{DHA-1}, and *qnrB4* in the same plasmid (Table 1) carried these genes on a genetic structure identical to that present in pKP048 (Fig. 1). However, pKP048 is an IncF plasmid of 150 kb in size, whereas S1 nuclease digestion (Promega, Madison, WI) and PFGE of the wild-type strains and transformants showed that these genes were borne by a plasmid of approximately 50 kb in the seven strains (data not shown). This plasmid was named pB1025, and it was confirmed to belong to the IncR family using a PCR-based replicon typing kit

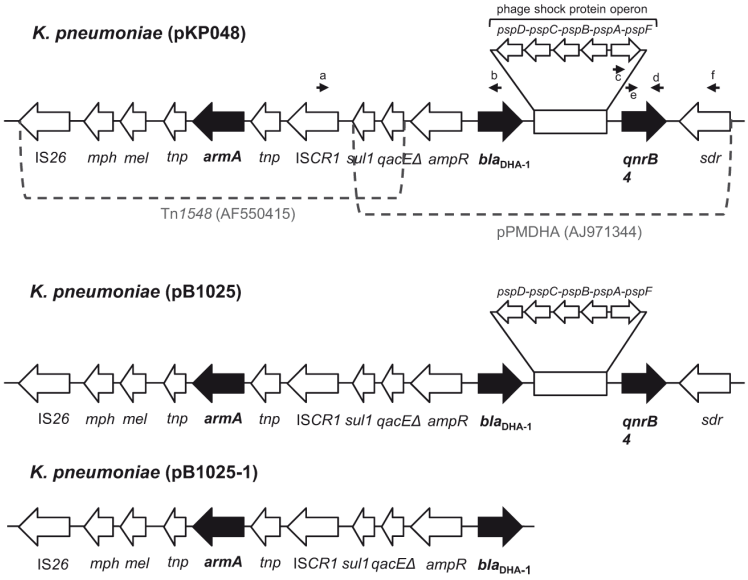

FIG 1 Illustration of the 25-kb genetic structure where *armA*, *bla*DHA-1, and *qnrB4* are embedded in pKP048 and comparison with plasmids pB1025 and pB1025-1. pB1025 has the same genetic structure as pKP048 despite being of a different size and belonging to another Inc family, whereas in pB1025-1, with the same size and Inc group as pB1025, *qnrB4* is not located on this genetic structure. The primers are indicated with small arrows: a, 5′ TCCAGACGGCCACATT GGAGG; b, 5′ TCAAATAGTGTATTTTCAGTG; c, 5′ CGATATCATGTTAATGGCTGA; d, 5′ GACGCCTTGCAAATCAACCCCG; e, 5′ CAGGTTCACCGGT GAAAAAGTT; f, 5′ ATCGCTGGCGAAGCAACTGGC.

(Diatheva, Fano, Italy). Interestingly, IncR plasmids have often been associated with human isolates, but to the best of our knowledge they have not been reported from pet animals to date. In strains BB1098 and BB1100, *armA* and bla_{DHA-1} are embedded in the same genetic structure as in the other five strains (Fig. 1). This genetic structure also takes part of an IncR plasmid of 50 kb, most likely a pB1025 derivative that was named pB1025-1, since *qnrB4* has a different environment and seems to be borne by a different plasmid. Attempts to conjugate pB1025 and pB1025-1 into a recipient laboratory strain as previously described (9) were unsuccessful.

This study describes the occurrence of the ArmA methyltransferase in an ST11 clone of *K. pneumoniae* isolated from pets in Spain, in association with the resistance genes bla_{DHA-1}, bla_{SHV-11}, and *qnrB4*. This is the first time that *armA* has been detected in bacteria from pet animals. To the best of our knowledge, this is also the first report of an IncR plasmid in bacteria isolated from pets. Interestingly, several reports from clinical settings point out that *K. pneumoniae* ST11 is a pathogenic clone adapted to humans and usually produces emerging resistance mechanisms. Thus, this finding is of utmost clinical relevance due to the relationship of pet animals with humans, as it poses a new reservoir for the dissemination of both the ST11 epidemic clone and these resistance genes. Further monitoring of emerging resistance genes in bacteria isolated from pets is essential to minimize their spread between humans and animals.

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DISCUSSION

1- Aminoglycoside resistance 16S rRNA methyltransferases: from the producer's necessity to the pathogen's advantage?

Acquired 16S rRNA MTs have recently emerged in gram-negative bacteria as a high-level resistance mechanism to aminoglycosides that mimics the one used by the aminoglycoside-producing bacteria to avoid the action of the antibiotic they generate. In the last decades, intrinsic 16S rRNA MTs have been thoroughly studied in aminoglycoside producers. Analysis of the known acquired MTs has revealed that they can be classified into two families, Arm and Pam, equivalent to the Kgm and Kam MTs families of the producers according to their methylation target in the 16S rRNA (Conn *et al.*, 2009). Nevertheless, the exact origin of acquired 16S rRNA MTs still remains uncertain.

Perhaps, based on the target of methylation it would be logical to assume that the acquired MTs originated from the intrinsic MTs. However, a few indicators make such a statement doubtful. When comparing the amino acid sequence of the acquired MTs with that of the chromosomally-encoded MTs from the producers, it can be seen that less than 33% identity exists between them. In addition, after the identification of several acquired MTs, it was observed that their overall G+C content was low (Figure 13) compared to the MT genes from the actinomycetes (ranging between 64% and 72%), which suggests that the acquired MTs are unlikely to be derived from those of the producers (Liou *et al.*, 2006). Gram-negative bacteria bearing an acquired MT usually display a genomic G+C content lower than that of the actinomycetes. Furthermore, the average G+C content of the close genetic environment of certain acquired MTs genes, such as *armA* and *rmtE*, is even higher than the MT gene G+C content (Galimand *et al.*, 2003; Davis *et al.*, 2010). However, genes with a G+C content close to that of their bacterial genome could have been integrated into it many years ago and adapted to the average total G+C content (Hayek, 2013), hence, the hypothesis that acquired MTs could originate from the producers cannot be entirely ruled out. It also has to be taken

into account that aminoglycoside resistance via methylation is not the only ribosomal target-based strategy found to avoid suicide in the producers (Cundliffe and Demain, 2010). Erm methyltransferases, responsible for macrolide resistance by methylating the 23S rRNA in the producers of macrolides (also actinomycetes), are expressed in a wide range of microorganisms including gram-positive species and phylogenetically remote gram-negative bacteria. It has been suggested that these macrolide resistance MTs in gram-positive (i.e. *Staphylococcus* spp.) and gram-negative (i.e. *E. coli*) bacteria are likely to have originated from a common ancestor in the producers a long time ago, despite the diversity of the codon usage (Arthur *et al.*, 1987). Regarding this option, it would be interesting to analyse the presence of acquired aminoglycoside resistance MTs in gram-negative bacteria isolated a long time ago (prior to the late 90s) as well as to compare the G+C content of their genes to that of the recently described acquired MT genes.

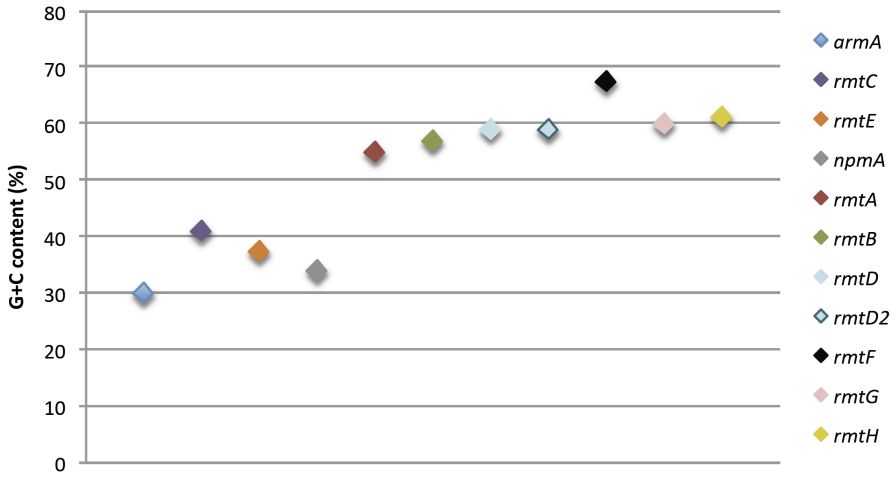

Figure 13. Chart showing the G+C content (%) of the acquired 16S rRNA MT genes reported to date.

Alternatively, the existence of gram-positive bacteria with a low genomic G+C content has been described, such as some species of the genus *Bacillus*, that produce small amounts of some aminoglycosides (i.e., *Bacillus circulans* naturally produces butirosin), making any of these producers a viable candidate as a source of acquired 16S rRNA MTs. For this reason, Liou *et al.* cloned and expressed

armA into *Bacillus subtilis* where it conferred a high-level aminoglycoside resistance (Liou *et al.*, 2006). Another recent study confirmed that RmtC confers the high-level aminoglycoside resistance phenotype when cloned into *B. subtilis* and *Staphylococcus aureus* (Wachino *et al.*, 2010). These assays further strengthened the hypothesis of low G+C content gram-positive bacteria as the origin of acquired MTs. Moreover, *B. circulans* is a bacterium found in soil that may be involved as an opportunistic pathogen in human infections. Thus, we wonder whether this microorganism is more related to gram-negative bacteria present in clinical settings and the commensal microbiota than to the environmental actinomycetes, which are rarely found to be pathogenic bacteria. To overcome this issue, it would be relevant to screen low G+C content aminoglycoside-producing bacteria, such as *Bacillus* spp, for possible aminoglycoside resistance MTs.

After considering that acquired AG resistance MTs might in fact derive from the producers, either the environmental Actinobacteria or the low G+C content gram-positive bacteria, a third option may be considered: could these MTs have originated from an endogenous 16S rRNA MT in gram-negative bacteria? It is well known that rRNA sequences adjacent to the nucleotides that interact with aminoglycosides at the 16S rRNA Helix 44, are targeted by several endogenous methyltransferases. These housekeeping modifications are believed to influence both the function and the structure of the ribosome (Decatur and Fournier, 2002), and these methyltransferases are highly conserved displaying a G+C content similar to that of the host bacterium (Gutierrez *et al.*, 2013). Therefore, would it be possible that faced with an increasing aminoglycoside selective pressure, gram-negative bacteria could have developed the ability of adapting an endogenous MT to methylate the residues G1405 or A1408 involved in aminoglycoside resistance? An in-depth comparative structural analyses of endogenous and acquired MTs would provide insight into the origin of acquired 16S rRNA MTs.

So far three possible origins of the acquired 16S rRNA MTs have been mentioned. However, we should not dismiss the idea of more than one origin, due to the low similarity between some of the acquired MTs. In any of these scenarios, all the acquired MTs could have evolved from a common ancestor or, on the

contrary, independently from each other. If we re-analyze the base usage of the acquired 16S rRNA MTs, it can be observed that the G+C content of *armA*, *rmtA-E*, and *npmA*, is significantly lower than that of the last three reported MT genes, *rmtF*, *rmtG*, and *rmtH* (Figure 13). While *rmtA*, *rmtB*, and *rmtD/D2* show a G+C content of 55-59%, similar to the average G+C content of many *Enterobacteriaceae*, the G+C content of *armA*, *rmtC*, *rmtE*, and *npmA* ranges from 30% to 41%, which is even lower than that of their close genetic environment. Interestingly, these numbers match the average genomic G+C content of several *Bacillus* spp. strains (including *B. circulans*). In addition, the dendrogram formed with the acquired G1405 16S rRNA MTs suggests that they are clearly divided into two groups where ArmA, RmtC and RmtE constitute the first group (Figure 14). On the other hand, the higher G+C content of the newly identified MT genes (*rmtF,*67.5%; *rmtG,*60%; *rmtH,*61.2%) approaches that of the environmental actinomycetes, suggesting for these a direct origin from the producers is likely.

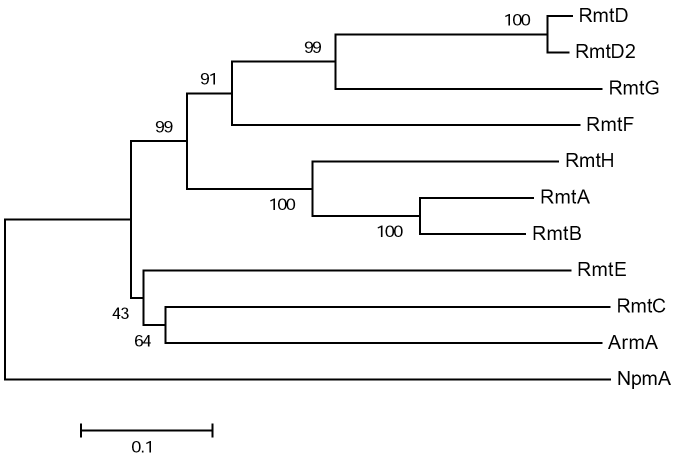

Figure 14. Dendrogram constructed with the acquired 16S rRNA MTs reported to date. The bar denotes genetic distance. Bootstrap values are the result of 1000 iterations.

In this work, the emergence and spread of the novel *rmtF* gene in India and the UK have been elucidated, as well as confirming the presence of *rmtF* in different plasmids and enterobacterial species. Thus, this MT possibly originated independently from other acquired MTs, jumped from the producers to *Enterobacteriaceae* some time ago and escaped unidentified. This is not surprising

if one takes into consideration that there were almost no screenings for acquired MTs in India prior to this study (where the strains were collected in 2010-2011). If at least some of these acquired MTs arose from an unrelated origin, this would indicate that novel acquired aminoglycoside resistance MTs might shortly emerge among clinical isolates.

2. Current prevalence and distribution of acquired 16S rRNA MTs: tying up loose ends

This work has mentioned how the emergence of acquired 16S rRNA MTs among gram-negative pathogens was a sudden process of rapid development, and the available data regarding the epidemiology of these enzymes is increasing substantially. However, there are several issues that remain unclear and open the door for a number of questions.

Regarding the prevalence of this resistance mechanism, it is interesting to point out that ArmA and RmtB are very highly prevalent compared to the other methyltransferases. This could be explainedby the enhanced dissemination facilitated by the mobile elements carrying these genes. Other acquired MTs have only been isolated from a few isolates. For example, the novel RmtH has only been detected in a single isolate, but it must be taken into consideration that this is a recent report and further publications might appear in the coming years. This is not the case for the only acquired MT belonging to the Pam family, NpmA, which was reported in 2007 from a single *E. coli* strain, and despite conferring pan-aminoglycoside resistance, it has not been isolated since then. NpmA confers resistance to 4,6-DOS, but also, a high-level resistance to the 4,5-DOS and apramycin (Wachino *et al.*, 2007). Given the little usage of the 4,5-DOS AGs as compared to the 4,6-DOS, and the lack of apramycin utilization in human medicine, we wonder whether this would be related to the cessation of both NpmA spreading and the identification of novel acquired MTs of the A1408 residue. As apramycin is a non-absorbed oral antibiotic used mainly for the treatment of colibacillosis, salmonellosis and enteritis in poultry and livestock, further

monitoring of N1-A1408 methyltransferases among veterinary samples would be pertinent. Indeed, this underlines antibiotic usage as a key factor for the dissemination and maintenance of resistance determinants.

RmtA prevalence and distribution is also worth mentioning. Regardless of its early identification, this acquired MT was only found in a few *P. aeruginosa isolates* from Japan, plus one *P. aeruginosa* strain from South Korea (Yokoyama *et al.*, 2003; Yamane *et al.*, 2007; Jin *et al.*, 2009), until it was described in a *K. pneumoniae* from an Indian patient (Poirel *et al.*, 2011b). Why have no reports of RmtA come up since then? In those *P. aeruginosa* strains where the *rmtA* genetic surroundings have been elucidated, the *rmtA* gene is found within a Tn*5041* variant. This transposon is well adapted to *P. aeruginosa*. Thus, it would be interesting both to further screen for the presence of *rmtA* in *P. aeruginosa* isolates as well as to determine the genetic environment of *rmtA* in *K. pneumoniae*. Perhaps this gene was spreading via the Tn*5041* transposon and once it passed to *K. pneumoniae*, the dissemination of this transposon was unsuccessful among any bacteria other than *P. aeruginosa*.

The evolution of RmtC dissemination after its first identification is also worth mentioning, as for a long time it was thought to be present only in those *Proteus mirabilis* strains found in Japan and Australia (Wachino *et al.*, 2006b; Zong *et al.*, 2008), so it did not pose a considerable threat for serious infections. Nevertheless, since its discovery in *Salmonella enterica* isolates from the USA and the UK (Folster *et al.*, 2009; Hopkins *et al.*, 2010), there has been a noticeable increase in the number of publications regarding the appearance of this acquired MT among different bacterial species (Poirel *et al.*, 2011c; Islam *et al.*, 2012; Williamson *et al.*, 2012). Interestingly, most of those *S. enterica* strains were isolated from patients that had recently travelled to India, and *rmtC* appeared to always be adjacent to an IS*Ecp1* mobile element. Furthermore, in this work, the presence of RmtC associated with RmtF and NDM-1 in Indian isolates is demonstrated, as well as in one UK isolate from a patient who had recently travelled to India. This geographical region seems to play a role as a reservoir for antibiotic resistance, especially if we take several factors into account, including

the lack of effective policies for the control of antibiotic usage. In addition, IS*Ecp1* is likely to be responsible for the transposition and spreading of *rmtC* (Wachino *et al.*, 2006a), therefore, we present a diagram (Figure 15) to explain and possibly predict the dissemination of RmtC, which has recently been increased and might be exponentially accelerated in the near future.

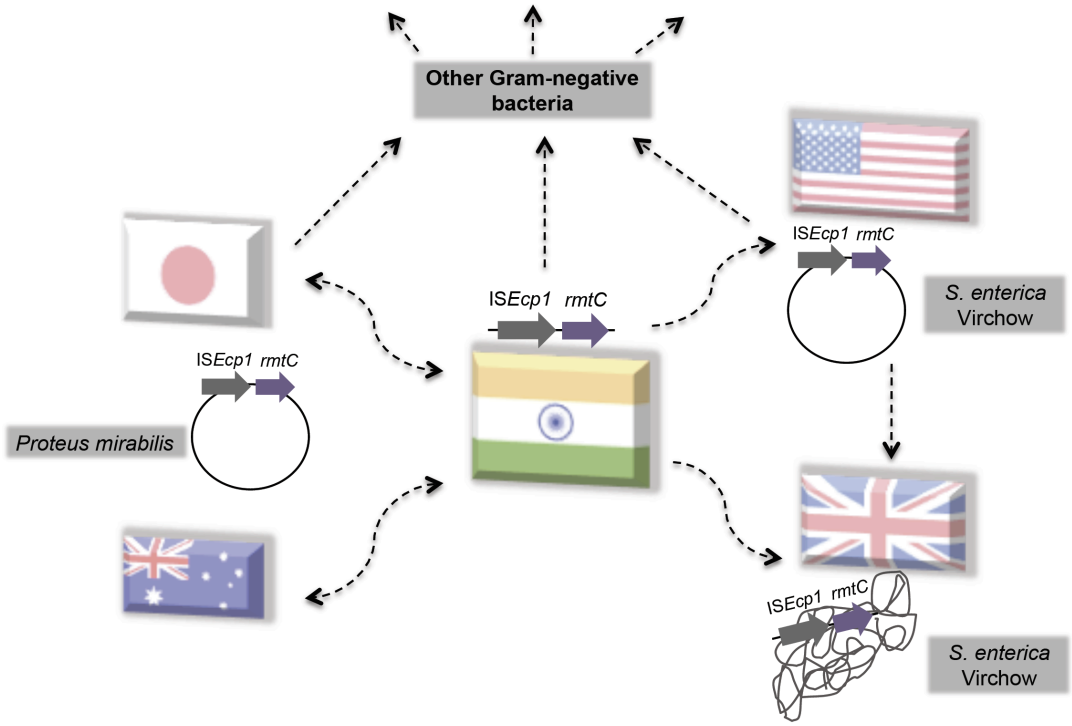

Figure 15. Diagram representing the potential past and future dissemination pathways of RmtC.

Similar events can be pointed out for each acquired 16S rRNA MT related to their geographical distribution. For instance, in a similar way as RmtA presence is limited to Asia, the acquired MTs RmtD and RmtD2 seem to be present only in South America, despite their first identification being a long time ago. RmtG, which has recently been found in Brazil (Bueno *et al.*, 2013) shares the highest similarity with RmtD, and both methyltransferases have almost the same G+C content (60%). This strongly suggests that the RmtD and RmtG origin and dissemination are likely related, and monitoring RmtG in gram-negative bacteria

worldwide in order to decipher whether its presence is confined to South America would be appropriate. The genetic environment of *rmtD* and *rmtD2* has been elucidated for some strains, and IS*CR14* may be involved in their dissemination. Thus, the lack of RmtD/D2 findings in the rest of the world is surprising, given the many migration movements that take place between continents nowadays. Most of the acquired MT producers are pathogenic bacteria isolated from inpatients and outpatients, but it is important to realize that many bacteria bearing an AG resistance MT might be undetected as they belong to the normal microbiota. Hence, we should consider the idea of also performing surveillance on healthy individuals as this would result in trustworthy data regarding the real prevalence and distribution of these resistance determinants.

These issues are a reminder that it is critical to not only detect new acquired MTs, but also to thoroughly analyze those traits associated with the known ones in order to gain the most knowledge. This will allow for a better understanding of the past, present, and possible future of this resistance mechanism.

3. Animals, food, and the environment: supporting actors or main characters in a never-ending story?

Since our group identified ArmA in an *Escherichia coli* of porcine origin right after its first publication, non-human sources were dragged into the spotlight as possible vehicles for the dissemination of acquired 16S rRNA MTs. However, there were not many studies in the first years on samples other than those from clinical settings.

This work has contributed to a better understanding of the role that animals and food play in the emergence of acquired MTs. Primarily, we have confirmed the presence of the most prevalent acquired MT, ArmA, in a bacterium isolated from food for the first time. We have also detected this MT in *K. pneumoniae* ST11 isolated from companion animals in Spain. RmtB was the only acquired MT previously found in bacteria collected from pets (Deng *et al.*, 2011a). These findings are noteworthy, as *K. pneumoniae* ST11 is a pathogenic clone adapted to

humans and related to MDR phenotypes. The low detection of acquired MTs among bacteria from pets led us to think that companion animals were not ultimately responsible for this resistance determinant spreading. However, the successful colonization of different pets by a human-adapted clone, such as *K. pneumoniae* ST11, gives rise to consider companion animals as a potential vector for bacteria highly resistant to AGs. This might be promoted by the tight relationship between humans and pets, but also by the usage of nearly the same aminoglycosides for the treatment of companion animals and humans.

Most findings of an acquired 16S rRNA MT from food, animals or the environment do not suggest that these sources are the main cause for the MTs dissemination. For instance, the RmtD-producing strain from a Brazilian river (Fontes *et al.*, 2011), and the RmtC positive *Samonella* isolated from food in the UK (Hopkins *et al.*, 2010), were clonally related to those human strains previously described to carry RmtD in Brazil and RmtC in the UK, respectively. Moreover, the ArmA-producing *S. enterica* strain from Reunion Island reported in this work, was recovered from chicken meat sampled during a control by the retailer in a supermarket. Interestingly, no other MDR strain was detected from farm chickens during a surveillance study performed at that time in Reunion Island (Henry *et al.*, 2009). This strongly suggests that these cases are the result of a cross contamination from a human origin. However, different studies have reported that the widespread dissemination of RmtB in China is not only associated with livestock and farm animals, but livestock-farming environments, farm workers, and pets (Chen *et al.*, 2007; Liu *et al.*, 2008; Du *et al.*, 2009; Deng *et al.*, 2011a). Moreover, the prevalence rate of RmtB-producing bacteria from animals is much higher than that from clinical strains (Deng *et al.*, 2013), contrary to the other acquired MTs. Around half of RmtB-positive isolates also harbour the fluoroquinolone resistance efflux pump QepA, and several studies have shown a link between both resistance determinants within the same mobile structure (Poirel *et al.*, 2012). Due to the high usage of quinolones in animals in the last decades, this could be promoting a co-selection for RmtB, and in this particular case, animal sources would be the main transmission vector of this acquired MT.

Therefore, depending on factors such as the association of different resistance determinants on the same mobile genetic structure, or the antibiotic stewardships in veterinary medicine, non-human sources could either be the principal vehicle of a particular 16S rRNA MT or just another route of transmission due to human contamination. Generally, it has been considered that food-producing animals play a more important role than pets in the transmission of antibiotic resistance. To clarify this, it would be interesting to sample owners of pets carrying MT-producing bacteria, or even pets of patients carrying MT-producing bacteria, in order to better ascertain the role of companion animals in the dissemination of antibiotic resistance.

4. Acquired 16S rRNA MTs in carbapenemase-producing bacteria (CPB): a global threat

We have previously mentioned that organisms producing acquired aminoglycoside resistance MTs are often multidrug resistant. Such bacteria currently pose one of the greatest risks to Public Health. The main antibiotic classes for the treatment of gram-negative bacterial infections are aminoglycosides, fluoroquinolones, and β-lactams. Therefore, the co-existence of mechanisms conferring resistance to these antibiotic families strongly limits the therapeutic options available. Moreover, the association of resistance determinants on the same plasmid is worrisome as they disseminate faster due to co-selection by different drugs.

Since their first identification, there has been a large association of AG resistance MTs with ESBLs, especially CTX-M-type enzymes (shown in Table 12 in the introduction section). Monotherapy with broad-spectrum β-lactams, such as cefotaxime, was commonly used to treat gram-negative bacterial infections, and aminoglycosides became an alternative for the treatment of infections caused by ESBL-producing organisms. Therefore, bacteria co-producing AG resistance MTs and ESBLs became of concern in the last years. However, carbapenem-hydrolyzing

β-lactamases, which slowly emerged in *Enterobacteriaceae* in the early 2000s, have recently become increasingly prevalent worldwide.

Table 13. Associations of AG resistance MTs and carbapenemases published to date

Acquired 16S rRNA MT	Carbapenemases	References
ArmA	OXA-23-like, VIM-2, IMP-1, KPC-2*, NDM-1*, OXA-24-like, OXA-58-like, VIM-4, VIM-1, OXA-48*	Doi *et al.*, 2007; Lee *et al.*, 2007; Kim *et al.*, 2008; Gurung *et al.*, 2010; Jiang *et al.*, 2010; Karthikeyan *et al.*, 2010; Zacharczuk *et al.*, 2010; Bogaerts *et al.*, 2011; Karah *et al.*, 2011; Ho *et al.*, 2011; Poirel *et al.*, 2011a; Samuelsen *et al.*, 2011; Sekizuka *et al.*, 2011; Solé *et al.*, 2011; Sung *et al.*, 2011; Dortet *et al.*, 2012; McGann *et al.*, 2012; Dolejska *et al.*, 2013; Tada *et al.*, 2013c, This work
RmtA	NDM-1, VIM-2	Jin *et al.*, 2009; Poirel *et al.*, 2011b
RmtB	OXA-23-like, OXA-51-like, KPC-2*, NDM-1, NDM-8	Galani *et al.*, 2012; Poirel *et al.*, 2011b; Sheng *et al.*, 2012; Tada *et al.*, 2013b, 2013c; This work
RmtC	NDM-1	Poirel *et al.*, 2011c; This work
RmtD/D2	SPM-1, KPC-2*	Doi *et al.*, 2007c, 2007d; Fontes *et al.*, 2011; Bueno *et al.*, 2013
RmtE	–	–
RmtF	NDM-1*	This work
RmtG	KPC-2*	Bueno *et al.*, 2013
RmtH	–	–
NpmA	–	–

* Carbapenemase and MT genes co-harbored by the same plasmid

The clinically most important carbapenemases in gram-negative pathogens are the class A enzymes of the KPC type, characteristic of MDR *K. pneumoniae*; the zinc-dependent class B metallo-β-lactamases (MBLs), represented mainly by the VIM, IMP, and NDM types; and the class D carbapenem-hydrolyzing OXA enzymes (Tzouvelekis *et al.*, 2012). As carbapenemases inactivate almost all

available β-lactams, if aminoglycoside activity is lost due to 16S rRNA methylation, we are facing a real threat with nearly no therapeutic tools. Fortunately, the appearance of AG resistance MTs within CPB was a relatively rare phenomenon until the extensive worldwide spread of the NDM-1 enzyme, firstly described in 2009, when it was repeatedly documented (Table 13). In fact, most publications discussing AG resistance 16S rRNA MTs in CPB are related to NDM-1 MBL. There is no characteristic association of a particular carbapenemase and an AG resistance MT with the exception of RmtD, which is generally described in SPM-1-producing *P. aeruginosa* from Brazil (Table 13).

Besides the importance of focusing on this emerging concern, it is necessary to not forget the relevance of ESBLs regardless of their high prevalence and dissemination, in particular the scarcely reported enzymes, such as the VEB ESBLS. These enzymes confer resistance to aztreonam, the only β-lactam not hydrolyzed by NDM-1. In this work, we have described the presence of a novel VEB enzyme variant, VEB-5, in clinical ArmA-producing *S. enterica* from the UK. Thus, resistance to AG and all β-lactams except carbapenems converge in highly pathogenic bacteria like *Salmonella* spp. Moreover, the *armA* and $bla_{\text{VEB-5}}$ genes were located on the same plasmid. This indicates a strong likelihood for the emergence of gram-negative pathogens resistant to all existing β-lactams due to the co-expression of NDM and VEB enzymes. Therefore it is crucial to simultaneously pay attention to the trend of associations between these resistance determinants.

In addition, with this work we have contributed to the deciphering of the growing emergence of AG resistance MTs in NDM-1-producing pathogens in India, one of the countries believed act as the epicentre of the NDM-1 epidemic (Livermore *et al.*, 2011b).

In our study, several enterobacteria isolated in the UK co-produce NDM-1 and the novel RmtF. Although this topic has raised controversy, there are some findings that clearly point out certain geographical regions of the Indian subcontinent as the origin for this emergence in the UK. Firstly, in the same year there is a higher prevalence of *rmtF*-bearing strains isolated from India than the UK. Out of the six UK isolates producing RmtF and NDM-1, four were isolated

from patients who had recently travelled to India, and out of these four strains, one shared 88% genetic similarity with an Indian isolate. Furthermore, *K.pneumoniae* isolates carrying NDM-1 and diverse AG methyltransferases, including RmtF, have just now been published (Tada *et al.*, 2013a). These bacteria were isolated from inpatients at a Teaching Hospital in Kathmandu, Nepal, in 2012. Interestingly, the RmtF-bearing bacteria from our study were collected during 2010 and 2011 at the Sanjay Gandhi Postgraduate Institute of Medical Sciences in Lucknow, India. This is a referral hospital that draws patients from most of the northern Indian states, mostly from the states of Uttar Pradesh and Bihar. These are the Indian regions geographically adjacent to Nepal (Figure 16).

Figure 16. Map showing the Indian states where the RmtF-bearing strains from this study were isolated.

The sequence of an NDM-1-bearing plasmid, pKPX-1 (AN: AP012055) was recently published, mentioning a *putative rRNA methyltransferase* in the sequence annotation, and it turns out that this plasmid contains an *rmtF* gene. This strain came from a Taiwanese patient with a hospitalization history in New Delhi, India. In these strains the immediate genetic environment downstream of *rmtF* consists of an *insE* transposase with a duplicated *oriIS* IS*CR5* upstream of the MT gene, as it was also found in our work. Therefore it seems that *rmtF* could be spreading out mainly through transposition by this mobile element. These results constitute more

evidence of what has recently been suspected, as it is frequent to detect NDM-1-positive strains from patients with travel history to the Indian subcontinent. In addition, the currently increasing human migration from- and to- countries with poor policies regulating antibiotic usage might support this hypothesis.

Unfortunately there are not many possibilities to control the flow of these bacteria between countries, hence, it is extremely necessary to resolve the root of the problem by controlling AB use in those countries. It is striking to observe that, despite the increasing number of reports regarding the association of acquired MTs and carbapenemases, so far none of these identified bacteria were collected from animals. Only very recently has the emergence of CPB from farm animals been reported (Seiffert *et al.*, 2013), possibly as a result of the reduced use of carbapenems in veterinary medicine. Currently the stage of carbapenem emergence in bacteria from animals resembles the early stages of ESBL dissemination, thus carbapenems should not be licensed for use in animals. However, the co-selection of CPB with other antibiotics used in veterinary medicine would still inflate the problem.

For this reason, it is not surprising that in 2012, the WHO focused its strategy on the five most important areas for the control of antibiotic resistance, which are: surveillance, rational use in humans, rational use in animals, infection prevention and control, and innovation. At the present stage, a global surveillance comprising the identification of known and novel resistance determinants, their molecular and epidemiological characterization, and the monitoring of resistance emergence to the latest therapeutic options, such as colistin and tigecycline, in MT-positive CPB, is decisive. Not for nothing, we are already facing this problem, as tigecycline and colistin resistance was detected in four and two *rmtF*-bearing isolates, respectively, in this study.

CONCLUSIONS/CONCLUSIONES

First. ArmA, the most prevalent aminoglycoside resistance methyltransferase, is present in *Salmonella enterica* isolated from chicken meat in Reunion Island. This is the first report of ArmA in East Africa and from bacteria collected from food, which implies a novel route of spread for this resistance determinant.

Primera. ArmA, la metiltransferasa de resistencia a aminoglucósidos más prevalente, está presente en *Salmonella enterica* aislada de carne de pollo en La Isla de Reunión. Esta es la primera identificación de ArmA en África del Este y en una bacteria de origen alimentario, lo que implica una nueva ruta de transmisión para este determinante de resistencia.

Second. We have developed a method consisting of nine overlapping PCRs to amplify Tn*1548,* the genetic platform in which the *armA* gene was originally detected. Tn*1548*-like structures seem to accelerate the spread of *armA* among different plasmids and gram-negative bacterial species.

Segunda. Hemos desarrollado un método de nueve PCRs solapantes para amplificar el Tn*1548*, la plataforma genética en la cual *armA* fue identificado por primera vez. Distintas variantes del Tn*1548* están acelerando la diseminación de *armA* a través de distintos plásmidos y especies de bacterias gram-negativas.

Third. We have identified a novel VEB enzyme, VEB-5, an ESBL responsible for high-level resistance to aztreonam in clinical *Salmonella* Worthington from the UK. Its coding gene is co-located with *armA* in pB1016, a conjugative IncA/C plasmid. This is the first association of ArmA with a VEB-type ESBL.

Tercera. Hemos identificado VEB-5, una nueva ESBL que confiere alto nivel de resistencia a aztreonam, en aislados clínicos de *Salmonella* Worthington de Reino Unido. El gen de esta nueva enzima está localizado junto con *armA* en pB1016, un plásmido conjugativo IncA/C. Esta es la primera asociación de ArmA con una BLEE de tipo VEB.

Fourth. RmtF is a new member of the 16S rRNA methyltransferases family that confers high-level resistance to aminoglycosides via the methylation of nucleotide G1405 in the 16S rRNA. This protein, of 260 amino acids, shares 46% amino acid identity with its closest methyltransferase, RmtD.

Cuarta. RmtF es un nuevo miembro de la familia de las metiltransferasas del ARNr 16S que confiere alto nivel de resistencia a aminoglucósidos al metilar el nucleótido G1405 en el ARNr 16S. Esta proteína, de 260 aminoácidos, comparte un 46% de identidad aminoacídica con la metiltransferasa más cercana, RmtD.

Fifth. The novel aminoglycoside resistance determinant, RmtF, is disseminating on diverse plasmids among clinical *Enterobacteriaceae* in India and the UK. These bacteria often co-produce a second methyltransferase as well as other emerging resistance mechanisms, such as NDM carbapenemase.

Quinta. El nuevo determinante de resistencia a aminoglucósidos RmtF, se está diseminando en Enterobacterias de origen clínico de India y de Reino Unido, localizado en diversos plásmidos. Estas bacterias a menudo expresan una segunda metiltransferasa, así como otros mecanismos de resistencia emergentes, como la carbapenemasa NDM.

Sixth. ArmA has been detected in bacteria isolated from companion animals for the first time. It is carried with QnrB4 and DHA-1 determinants in *Klebsiella pneumoniae* ST11, which is a human epidemic clone. This suggests the existence of a new reservoir for clinical multidrug-resistant *K. pneumoniae*.

Sexta. Hemos detectado ArmA por primera vez en bacterias aisladas de animales de compañía. Se trata de *Klebsiella pneumoniae* ST11, un clon epidémico en humanos que, además de ArmA, porta los determinantes QnrB4 y DHA-1. Este hallazgo supone un nuevo reservorio para *K. pneumoniae* multirresistentes de origen clínico.

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